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OPINION REVIEW

Reducing anastomotic leak in colorectal surgery: The old dogmas and the new challenges

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Abstract

Anastomotic leak (AL) constitutes a significant issue in colorectal surgery, and its incidence has remained stable over the last years. The use of intra-abdominal drain or the use of mechanical bowel preparation alone have been proven to be useless in preventing AL and should be abandoned. The role or oral antibiotics preparation regimens should be clarified and compared to other routes of administration, such as the intravenous route or enema. In parallel, preoperative antibiotherapy should aim at targeting collagenase-inducing pathogens, as identified by the microbiome analysis. AL can be further reduced by fluorescence angiography, which leads to significant intraoperative changes in surgical strategies. Implementation of fluorescence angiography should be encouraged. Progress made in AL comprehension and prevention might probably allow reducing the rate of diverting stoma and conduct to a revision of its indications.

Key words: Anastomotic leakage; Rectal surgery; Colic surgery; Prevention; Surgical site infection; Anastomosis; Complication

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Core tip: The present manuscript reviews the current evidence regarding the prevention of anastomotic leak in colorectal surgery. Oral antibiotics and fluorescence angiography might help reduce the incidence of anastomotic leak. Study of the microbiome might offer interesting paths for research. Progress made in anastomotic leak comprehension and prevention might allow reducing the rate of diverting stoma and conduct to a revision of its indications.

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INTRODUCTION

Anastomotic leak (AL) refers to the communication between hollow viscera lumen and the peritoneal cavity at the anastomotic level[1]. Despite a lack of widely accepted consensus regarding the definition of AL[2,3], AL was documented to occur in 8.1% of patients after right hemicolectomy according to the 2015 ESCP snapshot audit[4], 6.4% after colonic cancer surgery according to a nationwide Danish study including 9’333 patients[5] and 11% after rectal cancer surgery as reported by a systematic review and meta-analysis pooling 84 studies[6]. AL is graded according to the therapeutic management it requires: Grade A (no management), grade B (non operative management), grade C (operative management)[3].

In addition to the septic complications and prolonged hospitalization induced, AL leads to delayed adjuvant chemotherapy or no chemotherapy at all[7]. Further, a recent systematic review and meta-analysis including 78’434 colorectal cancer patients showed that AL was associated with increased local recurrence [risk ratio (RR) 1.90] and reduced overall survival (RR 1.36)[8]. Another systematic review and meta-analysis including 11'353 rectal cancer patients demonstrated that AL led to increased local recurrence [hazard ratio (HR) 1.71] and decreased survival (HR 1.67) and cancer-specific survival (HR 1.03) after anterior resection[9].

Despite the human and financial costs generated by AL[10], and the efforts put in reducing its occurrence, the incidence of AL has not evolved among the last years. Further, pre-operative prediction of AL and identification of at-risk patients are not accurate enough[11], and AL is often diagnosed too late in the postoperative period[12]. In an effort to optimize the therapeutic care of patients with colorectal anastomosis with the hope to reduce the occurrence of AL, we will review old dogmas regarding prevention of AL and confront them to the most recent evidence, and will define the new challenges in the field.

PREOPERATIVE MEASURES TO PREVENT AL

Patient-related factors

A recent systematic review identified several adjustable and non-adjustable risk factors for AL, including male gender, smoking, obesity, alcohol, steroid and non-steroidal anti-inflammatory drugs, operative time, transfusion, contamination of the operative field and emergency surgery[13]. Further, albumin < 3.5 g/dL, anemia, hypotension and use of inotropes were reported to increase the risk of AL[14]. Preoperative radiotherapy was also documented to constitute a risk factor for AL[15], especially if surgery occurred within an interval of 11-17 d after radiotherapy[16]. Therefore, adjustable risk factors should be corrected before proceeding to a digestive anastomosis, in order to reduce the risk of AL. This can be partly done through enhanced recovery programs[17], whose implementation in colorectal surgery units led to decreased postoperative morbidity and length of stay[18]. However, the effect of enhanced recovery protocols on the rate of AL remains to be demonstrated.

Preoperative oral antibiotics and mechanical bowel preparation

Preoperative mechanical bowel preparation alone has lost in interest after multiple publications demonstrating its absence of benefit in reducing AL in elective colorectal surgery. Contant *et al*[19] randomized 1’431 patients to receive or not mechanical bowel preparation before elective colorectal surgery, and showed that patients who received mechanical bowel preparation did not have a lower rate of AL. Further, a systematic review and meta-analysis of randomized clinical trial (RCT) including 4’859 patients confirmed this finding[20].

Recently, authors, such as Scarborough[21], postulated that mechanical bowel preparation allowed to improve the delivery of oral antibiotic preparation to the bowel mucosa and could therefore not be assessed independently. Using the American College of Surgeons National Surgical Quality Improvement Program

database and including 4'999 patients, they showed that combined preoperative oral antibiotics and mechanical bowel preparation lowered the rate of AL from 5.7% to 2.8% in colorectal surgery when compared to patients not receiving any kind of preparation. However, neither oral antibiotics alone or MBP alone allowed to lower the rate of AL[21]. Further publications reached the same conclusions but used the same database[22,23]. However, latest studies using the same database only showed an effect of oral antibiotics alone and demonstrated that combination with MBP offered no additional advantage[24,25].

Therefore, a large RCT is needed to determine whether oral antibiotics alone and intravenous antibiotics are sufficient in reducing AL after colorectal surgery or whether association with mechanical bowel preparation is needed[26]. Furthermore, the type of MBP is very different among surgeon practice[27] and probably needs standardization before conclusion could be drawn from MBP studies. The use of rectal enema associated or not with antibiotics should be assessed; as evidence is growing that the local microbiome at the anastomotic site might be implicated in AL, as discussed below.

Microbiology

Recent evidence supports the hypothesis that AL might result from a local infective complication, resulting in impaired healing at the anastomotic level due to a local increase in collagenase activity. For instance, Shogan *et al*[28] showed in a rat model that *Enterococcus faecalis* led to the degradation of collagen IV at the anastomotic level through activation of MMP9. Further, topical antibiotherapy administered by enema targeting *Enterococcus faecalis* allowed to reduce AL to 0%, whereas intramuscular cefotixim—commonly used for elective surgery prophylaxis—did not reduce collagenase activity nor AL[28]. Moreover, a recent study using rat model of colo-colic anastomosis demonstrated that the selective MMP-8, MMP-9, and MMP-12 inhibitor AZD3342 allowed to maintain the anastomotis baseline breaking strength and to reduce AL[29]. Butyrate, a short-chain fatty acid, was shown to reduce AL[30-32], probably through its inhibitory effect on *Pseudomonas aeruginosa*[33]. In a case-control study including 8 patients with AL and 8 patients without AL after stapled colorectal anastomosis, van Praagh *et al*[34] showed that patients with AL had lower microbial diversity and higher abundance of *Lachnospiraceae*. They postulated that the higher rate of AL might be explained by the presence of mucin-degrading *Ruminococci* within that family[34]. Stumpf *et al*[35] found lower collagen type I/III ratio and higher expression of MMP-1, -2 and -9 in biopsies of patients with impaired anastomotic healing when compared to controls. These results suggest that unfavorable microbiome comprising collagenase-inducing pathogens might impair anastomotic healing and result in AL.

Further, anastomosis creation was shown to result in a 200- and 500-fold increase in the relative abundance of Enterococcus and Escherichia/Shigella, respectively, in a rodent model[36]. In a prospective multicentric cohort of patients undergoing colorectal surgery, including our center, Dubinsky-Pertzov *et al*[37] showed that carriers of beta lactamase-producing *Enterobacteriaceae* receiving cephalosporin-based antibioprophylaxis were at risk of surgical site infection [odds ratio (OR) 2.36]. These findings suggest that changes in the local microbiome caused by surgery or unappropriate prophylactic antibiotherapy might worsen the situation of patients with already unfavorable microbiome profiles. Also, radiotherapy was documented to change the composition of the microbiome[38,39], which constitutes a finding of importance for rectal cancer patients receiving neoadjuvant treatment but with no clear demonstration in increased AL so far.

Microbiome is a new and very promising field of research, especially when studying the aetiologies of AL in colorectal surgery. Identifying at risk patients with unfavorable microbiome, comprising pathogens with high collagenase activity, and treating them with appropriate antibiotic regimen (per os, intravenous or by enema) and/or faecal transplantation if required could help reducing AL rate. Further, studying the microbiome might help explaining the protective effect of preoperative oral antibiotics on the AL rate.

OPERATIVE MEASURES TO PREVENT AL

Surgical approach

The United States Nationwide Inpatient Sample database (including 244'129 elective colectomies) was analyzed to compare outcomes between robot-assisted colectomy (1'584 colectomies), laparoscopic colectomy (116'261 colectomies) and open colectomy (126'284 colectomies). AL was not reported, but the authors described laparoscopic

colectomy to lower the risk of complications (19.8% *vs* 33.2%) and stoma (3.5% *vs* 13.0%) when compared to the open approach. No difference could be found between laparoscopy and the robotic technique regarding these outcomes[40]. On the opposite, analysis of the The Danish Colorectal Cancer Group database described laparoscopic colectomy as a risk factor for AL (OR 1.34) in 9'333 patients[5]. Regarding right colectomies, a systematic review and meta-analysis including 7'780 patients found no difference in terms of AL between the laparoscopic and robotic approaches[41]. Regarding elective and emergency sigmoidectomy for diverticulitis, a Cochrane systematic review and meta-analysis of RCT did not describe any difference in terms of reoperation due to AL between patients with laparoscopic colectomy and those with open colectomy (349 pooled patients for that outcome)[42]. Further, the intermediate analysis of the ROLARR trial described no difference in AL rate between the two approaches for rectal cancer[43]. Regarding the latest, the transanal total mesorectum excision (taTME) technique, bypassing the anatomic limitations of the narrow pelvis, might allow to reduce AL, but remains to be evaluated for that outcome.

Anastomosis technique

Handsewn and stapled anastomoses are still widely performed according to surgeons preferences, reflecting the lack of consensus regarding the anastomotic method. A Cochrane systematic review and meta-analysis including 1'233 patients from 9 RCT found no difference in terms of AL, clinical AL and radiological AL between patients with stapled or handsewn colorectal anastomoses[44]. However, the authors did not perform subgroup analysis according to the underlying disease or to the presence or not of associated procedures (drainage, diverting stoma). Further, all included studies were anterior to 1995. In emergency procedures, another systematic review and meta-analysis did not identify any statistical differences between stapled and handsewn anastomoses (1'120 patients)[45].

Regarding right colectomy or ileo-cacecal resection, the 2015 ESCP audit described an AL rate of 8.1% among 3'208 patients. After adjustment for confounding factors, the use of a stapler was significantly associated with AL (OR 1.43)[4]. Further, stapled anastomoses were more frequently used in low risk patients, resulting in a likely underestimation of the risk of AL after right colectomy or ileo-caecal resection.

We should note that lower anastomoses are more at risk of AL, as known since decades[15,46]. A snapshot audit specifically concerning left colon, sigmoid and rectal resections is currently undergoing[47] and therefore conclusion cannot be reached regarding left colon and rectal surgery. No evidence is supporting either of the construction methods used for colorectal anastomosis (side to side, end to side, side to end, end to end). The evidence seems to be more straightforward regarding the number of catridges used for rectal division. A retrospective study from Austria demonstrated in 382 patients who benefited from rectal division using a linear stapler and colorectal anastomosis using a circular stapler or compression device, that the use of 3 or more cartridges increased the incidence of AL (19.4% AL in this subgroup)[48]. Further, the number of intersections of staple lines also correlated to the rate of AL in colorectal anastomosis using a double stapling technique[49]. A single stapling technique for colorectal anastomosis (in TaTME for example), in opposition with the conventional double stapling technique, was demonstrated to be safe in low anterior resection but lacks evidences in term of reduction of AL[50].

Compression anastomosis consists of a stapler equipped with disposable rings used for colorectal anastomosis: The rings are applied on each side at the anastomotic level and are evacuated into the stools once tissue necrosis and healing have occurred. A study performed in pigs with colorectal anastomoses showed that compression anastomosis was associated with less inflammation and scarring when compared with the stapling technique[51]. A retrospective multicentric study including 1'180 patients described an AL rate of 3.22% using the ColonRing device[52]. Further, a prospective postmarketing evaluation of the ColonRing described an AL rate of 5.3% among 266 patients, but a septic anastomotic complication rate of 8.3%, which could reflect the true AL rate[53]. A recent systematic review and meta-analysis including 10 RCT (1'969 patients) found no difference in terms of AL between patients with handsewn or stapled anastomosis (977 patients) and those with compression anastomoses (992 patients)[54]. Compression anastomosis has however not gained in popularity.

Intraoperative assessment of the anastomosis

As previously reported[55], many methods have been develop to perioperatively assess the integrity of colorectal anastomoses. Briefly, the air leak test, which consists in insufflating the bowel at the anastomotic level to detect any AL, was demonstrated to help identifying AL perioperatively and led to their repair, resulting in lower rate of postoperative AL[56,57]. Intraoperative endoscopy, in addition, to evaluate the

anastomosis integrity, allows identifying bleeding at the anastomotic level or disruption of the anastomosis[58]. However, it requires endoscopy skills, extra material, is time-consuming and requires further scientific validation in terms of AL prevention[59].

New methods rely on the assessment of the blood supply to the anastomosis. Adequate perfusion of the healing tissue is key to prevent AL, and a reduction in the blood flow at the rectal stump was shown to correlate with AL[60]. Historical methods include relying on the color of the bowel, as proposed by Goligher[61], or observing the pulsatile flow at the cut section, as stated by Novell and Lewis[62]. Objective and reliable methods assessing anastomosis vascularization have been developed since, as reported in our recent review[55], mentioning notably Doppler ultrasound[63] and light spectroscopy[64]. More recently, fluorescence perfusion angiography has showed a widespread clinical use. Briefly, a fluorophore is injected intravenously, excited by a specific wavelength to emit in another specific wavelength (usually infrared) just after vessel division an/or completion of the anastomosis, allowing the surgeon to identify any defect in vascularization at the anastomotic level. Jafari *et al*[65] reported that fluorescence perfusion angiography allowed to reduce AL from 18% to 6% after robotic–assisted anterior resection. Using a prospective cohort of 504 patients undergoing elective colorectal surgery with anastomosis, our team demonstrated that fluorescence perfusion angiography allowed for a change in the strategy of bowel division due to insufficient perfusion in 5.8% of patients, with no subsequent AL[66]. Results of the PILLAR II study documented a change in the surgical plan in 8% of 139 included patients undergoing anterior resection with no subsequent AL in those patients[67]. A recent systematic review and meta-analysis pooling 1’302 patients confirmed these results by reporting that fluorescence perfusion angiography reduced the rate of AL in patients operated for colorectal cancer[68].

Therefore, old methods allowing assessing the integrity of the anastomosis and the absence of AL should be combined to new technologies, such as fluorescence perfusion angiography, which aim at determining the vascularization of the anastomosis, a prerequisite to an efficient healing process without subsequent AL. New studies should aim at determining whether stimulation of the neoangiogenesis process, for example by the local administration of recombinant VEGF[69], could help in further reducing the occurrence of AL.

Diverting stoma

The creation of a lateral ileostomy or colostomy in patients at risk of AL aims at diverting the bowel content away from the anastomosis in order to decrease the rate of AL and the related morbidity. However, diverting stoma expose the patients to the risk of dehydration or to stoma closure-related complications. Further, they lead to an additional scare or won’t be closed in a significant proportion of patients[70].

A Cochrane systematic review and meta-analysis including RCT assessing the use of prophylactic stoma versus no stoma in patients with low anterior resection for rectal cancer until November 2009 described the use of covering stoma to lower the incidence of AL (RR 0.33)[71]. Thereafter, a review of 525 patients with colo-anal anastomosis from the NSQIP database identified the absence of stoma as a risk factor for developing postoperative sepsis (OR 6.29), although the rate of AL was not reported. Also, allocation to the stoma group was not randomized and the effect was not observed in patients with low pelvic anastomosis (1’266 patients)[72]. A systematic review and meta-analysis including all studies published between 2014 and 2017 regarding the role of a protective stoma in patients undergoing low anterior resection, identified the presence of a stoma as a protective factor against AL (RR 0.38, 5’612 patients, 11 studies)[73]. A later systematic review and meta-analysis including only RCT (4 RCT, 358 patients) confirmed that diverting stoma lowers the risk of AL (OR 0.32)[74]. The Cochrane collaboration produced a systematic review and meta-analysis pooling 648 patients from 6 RCT and identified diverting stoma as a protective factor against clinical AL (RR 0.33) after low anterior resection[71].

Evidence regarding “ghost ileostomy” – a bowel loop brought through the abdominal wall but left unopened, leaving the possibility to be transformed in an ileostomy if needed – is low and remains to be clarified[3]. Therefore, we can conclude that diverting stoma allows reducing the occurrence of AL in at-risk patients (those with low anastomosis). Ghost ileostomy could constitute a solution to avoid the occurrence of stoma-related complications, but it should be keep in mind that ghost ileostomy won’t allow to avoid AL but rather to decrease its morbidity.

Prophylactic intra-abdominal drainage

Prophylactic intra-abdominal drainage during elective colorectal surgery was thought to help monitoring the occurrence of AL and to reduce its morbidity by avoiding a generalized peritonitis. The GRECCAR 5 trial compared 236 randomized rectal cancer

patients allocated to the intra-abdominal drain group to 233 patients allocated to the group without drainage. Intra-abdominal drainage did not allow to reduce the rate of pelvic sepsis, the postoperative morbidity, the reoperation rate, the lenght of hospital stay and the rate of stoma closure[75]. Later, a systematic review and meta-analysis pooling 760 patients from 4 RCT demonstrated that intra-abdominal drainage did not reduce AL, pelvic complications, reintervention and mortality. Contrariwise, the incidence of postoperative bowel obstruction was significantly higher in the drained group (OR 1.61)[76]. A Cochrane systematic review obtained the same conclusion thtat prophylactic intra-abdominal drainage did not reduce the rate of AL[77]. Therefore, prophylactic intra-abdominal drainage should be discouraged in elective colorectal surgery.

Prophylactic transanal tube decompression

Prophylactic transanal tube decompression was thought to lower the risk of AL whilst presenting less risks of complication that diverting stoma. A systematic review and meta-analysis pooling 1'772 patients undergoing anterior resection described transanl tube decompression to lower the risk of AL (RR 0.44)[78]. However, patients receiving diverting stoma were excluded, leading to a potential underestimation of the AL rate. Another systematic review and meta-analysis followed, including patients with diverting stoma, and obtained the same conclusion (a reduction of the risk of AL (RR 0.42) in patients with transanal tube decompression)[79]. Therefore, prophylactic transanal tube decompression could constitute an efficient method to prevent AL in high risk patients without exposing them to the complications of diverting stoma. A well-conducted large scale RCT comparing the 2 techniques remains, however, to be conducted.

CONCLUSION

AL still constitutes a significant issue in colorectal surgery, and its incidence has remained stable over the last years. The use of intra-abdominal drain or the use of mechanical bowel preparation alone have been proven to be useless in preventing AL and should be abandoned. The role or oral antibiotics preparation regimens should be clarified and compared to other routes of administration, such as the intravenous route or enema. In parallel, the composition of the microbiome of patients with AL should be precisely determined, in order to identify patients at risk of AL and offer targeted preoperative antibiotics. AL can be further reduced by fluorescence angiography, which leads to significant intraoperative changes in surgical strategies. Implementation of fluorescence angiography should be encouraged. Progress made in AL comprehension and prevention might probably allow reducing the rate of diverting stoma and conduct to a revision of its indications.

REFERENCES

1 **Rahbari NN**, Weitz J, Hohenberger W, Heald RJ, Moran B, Ulrich A, Holm T, Wong WD, Tiret E, Moriya Y, Laurberg S, den Dulk M, van de Velde C, Büchler MW. Definition and grading of anastomotic leakage following anterior resection of the rectum: A proposal by the International Study Group of Rectal Cancer. *Surgery* 2010; **147**: 339-351 [PMID: 20004450 DOI: 10.1016/j.surg.2009.10.012]

2 **Bruce J**, Krukowski ZH, Al-Khairy G, Russell EM, Park KG. Systematic review of the definition and measurement of anastomotic leak after gastrointestinal surgery. *Br J Surg* 2001; **88**: 1157-1168 [PMID: 11531861 DOI: 10.1046/j.0007-1323.2001.01829.x]

3 **Vallance A**, Wexner S, Berho M, Cahill R, Coleman M, Haboubi N, Heald RJ, Kennedy RH, Moran B, Mortensen N, Motson RW, Novell R, O'Connell PR, Ris F, Rockall T, Senapati A, Windsor A, Jayne DG. A collaborative review of the current concepts and challenges of anastomotic leaks in colorectal surgery. *Colorectal Dis* 2017; **19**: O1-O12 [PMID: 27671222 DOI: 10.1111/codi.13534]

4 **2015 European Society of Coloproctology collaborating group**. The relationship between method of anastomosis and anastomotic failure after right hemicolectomy and ileo-caecal resection: An international snapshot audit. *Colorectal Dis* 2017 [PMID: 28263043 DOI: 10.1111/codi.13646]

5 **Krarup PM**, Jorgensen LN, Andreasen AH, Harling H; Danish Colorectal Cancer Group. A nationwide study on anastomotic leakage after colonic cancer surgery. *Colorectal Dis* 2012; **14**: e661-e667 [PMID: 22564292 DOI: 10.1111/j.1463-1318.2012.03079.x]

6 **Paun BC**, Cassie S, MacLean AR, Dixon E, Buie WD. Postoperative complications following surgery for rectal cancer. *Ann Surg* 2010; **251**: 807-818 [PMID: 20395841 DOI: 10.1097/SLA.0b013e3181dae4ed]

7 **Smith JD**, Paty PB, Guillem JG, Temple LK, Weiser MR, Nash GM. Anastomotic leak is not associated with oncologic outcome in patients undergoing low anterior resection for rectal cancer. *Ann Surg* 2012; **256**: 1034-1038 [PMID: 22584695 DOI: 10.1097/SLA.0b013e318257d2c1]

8 **Ha GW**, Kim JH, Lee MR. Oncologic Impact of Anastomotic Leakage Following Colorectal Cancer Surgery: A Systematic Review and Meta-Analysis. *Ann Surg Oncol* 2017; **24**: 3289-3299 [PMID: 28608118 DOI: 10.1245/s10434-017-5881-8]

9 **Wang S**, Liu J, Wang S, Zhao H, Ge S, Wang W. Adverse Effects of Anastomotic Leakage on Local

Recurrence and Survival After Curative Anterior Resection for Rectal Cancer: A Systematic Review and Meta-analysis. *World J Surg* 2017; **41**: 277-284 [PMID: 27743072 DOI: 10.1007/s00268-016-3761-1]
10 **Ashraf SQ**, Burns EM, Jani A, Altman S, Young JD, Cunningham C, Faiz O, Mortensen NJ. The economic impact of anastomotic leakage after anterior resections in English NHS hospitals: Are we adequately remunerating them? *Colorectal Dis* 2013; **15**: e190-e198 [PMID: 23331871 DOI: 10.1111/codi.12125]
11 **Karliczek A**, Harlaar NJ, Zeebregts CJ, Wiggers T, Baas PC, van Dam GM. Surgeons lack predictive accuracy for anastomotic leakage in gastrointestinal surgery. *Int J Colorectal Dis* 2009; **24**: 569-576 [PMID: 19221768 DOI: 10.1007/s00384-009-0658-6]
12 **Hyman N**, Manchester TL, Osler T, Burns B, Cataldo PA. Anastomotic leaks after intestinal anastomosis: It's later than you think. *Ann Surg* 2007; **245**: 254-258 [PMID: 17245179 DOI: 10.1097/01.sla.0000225083.27182.85]
13 **McDermott FD**, Heeney A, Kelly ME, Steele RJ, Carlson GL, Winter DC. Systematic review of preoperative, intraoperative and postoperative risk factors for colorectal anastomotic leaks. *Br J Surg* 2015; **102**: 462-479 [PMID: 25703524 DOI: 10.1002/bjs.9697]
14 **Choudhuri AH**, Uppal R, Kumar M. Influence of non-surgical risk factors on anastomotic leakage after major gastrointestinal surgery: Audit from a tertiary care teaching institute. *Int J Crit Illn Inj Sci* 2013; **3**: 246-249 [PMID: 24459621 DOI: 10.4103/2229-5151.124117]
15 **Pommergaard HC**, Gessler B, Burcharth J, Angenete E, Haglind E, Rosenberg J. Preoperative risk factors for anastomotic leakage after resection for colorectal cancer: A systematic review and meta-analysis. *Colorectal Dis* 2014; **16**: 662-671 [PMID: 24655784 DOI: 10.1111/codi.12618]
16 **Pettersson D**, Cedermark B, Holm T, Radu C, Påhlman L, Glimelius B, Martling A. Interim analysis of the Stockholm III trial of preoperative radiotherapy regimens for rectal cancer. *Br J Surg* 2010; **97**: 580-587 [PMID: 20155787 DOI: 10.1002/bjs.6914]
17 **Carmichael JC**, Keller DS, Baldini G, Bordeianou L, Weiss E, Lee L, Boutros M, McClane J, Feldman LS, Steele SR. Clinical Practice Guidelines for Enhanced Recovery After Colon and Rectal Surgery From the American Society of Colon and Rectal Surgeons and Society of American Gastrointestinal and Endoscopic Surgeons. *Dis Colon Rectum* 2017; **60**: 761-784 [PMID: 28682962 DOI: 10.1097/DCR.0000000000000883]
18 **Greco M**, Capretti G, Beretta L, Gemma M, Pecorelli N, Braga M. Enhanced recovery program in colorectal surgery: A meta-analysis of randomized controlled trials. *World J Surg* 2014; **38**: 1531-1541 [PMID: 24368573 DOI: 10.1007/s00268-013-2416-8]
19 **Contant CM**, Hop WC, van't Sant HP, Oostvogel HJ, Smeets HJ, Stassen LP, Neijenhuis PA, Idenburg FJ, Dijkhuis CM, Heres P, van Tets WF, Gerritsen JJ, Weidema WF. Mechanical bowel preparation for elective colorectal surgery: A multicentre randomised trial. *Lancet* 2007; **370**: 2112-2117 [PMID: 18156032 DOI: 10.1016/S0140-6736(07)61905-9]
20 **Slim K**, Vicaut E, Launay-Savary MV, Contant C, Chipponi J. Updated systematic review and meta-analysis of randomized clinical trials on the role of mechanical bowel preparation before colorectal surgery. *Ann Surg* 2009; **249**: 203-209 [PMID: 19212171 DOI: 10.1097/SLA.0b013e318193425a]
21 **Scarborough JE**, Mantyh CR, Sun Z, Migaly J. Combined Mechanical and Oral Antibiotic Bowel Preparation Reduces Incisional Surgical Site Infection and Anastomotic Leak Rates After Elective Colorectal Resection: An Analysis of Colectomy-Targeted ACS NSQIP. *Ann Surg* 2015; **262**: 331-337 [PMID: 26083870 DOI: 10.1097/SLA.0000000000001041]
22 **Kiran RP**, Murray AC, Chiuzan C, Estrada D, Forde K. Combined preoperative mechanical bowel preparation with oral antibiotics significantly reduces surgical site infection, anastomotic leak, and ileus after colorectal surgery. *Ann Surg* 2015; **262**: 416-25; discussion 423-5 [PMID: 26258310 DOI: 10.1097/SLA.0000000000001416]
23 **Althumairi AA**, Canner JK, Pawlik TM, Schneider E, Nagarajan N, Safar B, Efron JE. Benefits of Bowel Preparation Beyond Surgical Site Infection: A Retrospective Study. *Ann Surg* 2016; **264**: 1051-1057 [PMID: 26727098 DOI: 10.1097/SLA.0000000000001576]
24 **Garfinkle R**, Abou-Khalil J, Morin N, Ghitulescu G, Vasilevsky CA, Gordon P, Demian M, Boutros M. Is There a Role for Oral Antibiotic Preparation Alone Before Colorectal Surgery? ACS-NSQIP Analysis by Coarsened Exact Matching. *Dis Colon Rectum* 2017; **60**: 729-737 [PMID: 28594723 DOI: 10.1097/DCR.0000000000000851]
25 **Koller SE**, Bauer KW, Egleston BL, Smith R, Philp MM, Ross HM, Esnaola NF. Comparative Effectiveness and Risks of Bowel Preparation Before Elective Colorectal Surgery. *Ann Surg* 2018; **267**: 734-742 [PMID: 28151800 DOI: 10.1097/SLA.0000000000002159]
26 **Beyer-Berjot L**, Slim K. Colorectal surgery and preoperative bowel preparation: Aren't we drawing hasty conclusions? *Colorectal Dis* 2018; **20**: 955-958 [PMID: 30199608 DOI: 10.1111/codi.14415]
27 **Devane LA**, Proud D, O'Connell PR, Panis Y. A European survey of bowel preparation in colorectal surgery. *Colorectal Dis* 2017; **19**: O402-O406 [PMID: 28975694 DOI: 10.1111/codi.13905]
28 **Shogan BD**, Belogortseva N, Luong PM, Zaborin A, Lax S, Bethel C, Ward M, Muldoon JP, Singer M, An G, Umanskiy K, Konda V, Shakhsheer B, Luo J, Klabbers R, Hancock LE, Gilbert J, Zaborina O, Alverdy JC. Collagen degradation and MMP9 activation by Enterococcus faecalis contribute to intestinal anastomotic leak. *Sci Transl Med* 2015; **7**: 286ra68 [PMID: 25947163 DOI: 10.1126/scitranslmed.3010658]
29 **Krarup PM**, Eld M, Jorgensen LN, Hansen MB, Ågren MS. Selective matrix metalloproteinase inhibition increases breaking strength and reduces anastomotic leakage in experimentally obstructed colon. *Int J Colorectal Dis* 2017; **32**: 1277-1284 [PMID: 28717842 DOI: 10.1007/s00384-017-2857-x]
30 **Rolandelli RH**, Buckmire MA, Bernstein KA. Intravenous butyrate and healing of colonic anastomoses in the rat. *Dis Colon Rectum* 1997; **40**: 67-70 [PMID: 9102264 DOI: 10.1007/BF02055684]
31 **Mathew AJ**, Wann VC, Abraham DT, Jacob PM, Selvan BS, Ramakrishna BS, Nair AN. The effect of butyrate on the healing of colonic anastomoses in rats. *J Invest Surg* 2010; **23**: 101-104 [PMID: 20497012 DOI: 10.3109/08941930903469367]
32 **Bloemen JG**, Schreinemacher MH, de Bruine AP, Buurman WA, Bouvy ND, Dejong CH. Butyrate enemas improve intestinal anastomotic strength in a rat model. *Dis Colon Rectum* 2010; **53**: 1069-1075 [PMID: 20551761 DOI: 10.1007/DCR.0b013e3181d881b7]
33 **Levison ME**. Effect of colon flora and short-chain fatty acids on growth in vitro of Pseudomonas aeruginsoa and Enterobacteriaceae. *Infect Immun* 1973; **8**: 30-35 [PMID: 4198102 DOI: 10.1007/BF01639659]
34 **van Praagh JB**, de Goffau MC, Bakker IS, Harmsen HJ, Olinga P, Havenga K. Intestinal microbiota and

anastomotic leakage of stapled colorectal anastomoses: A pilot study. *Surg Endosc* 2016; **30**: 2259-2265 [PMID: 26385781 DOI: 10.1007/s00464-015-4508-z]

35 **Stumpf M**, Klinge U, Wilms A, Zabrocki R, Rosch R, Junge K, Krones C, Schumpelick V. Changes of the extracellular matrix as a risk factor for anastomotic leakage after large bowel surgery. *Surgery* 2005; **137**: 229-234 [PMID: 15674206 DOI: 10.1016/j.surg.2004.07.011]

36 **Shogan BD**, Smith DP, Christley S, Gilbert JA, Zaborina O, Alverdy JC. Intestinal anastomotic injury alters spatially defined microbiome composition and function. *Microbiome* 2014; **2**: 35 [PMID: 25250176 DOI: 10.1186/2049-2618-2-35]

37 **Dubinsky-Pertzov B**, Temkin E, Harbarth S, Fankhauser-Rodriguez C, Carevic B, Radovanovic I, Ris F, Kariv Y, Buchs NC, Schiffer E, Cohen Percia S, Nutman A, Fallach N, Klausner J, Carmeli Y; R-GNOSIS WP4 study group. Carriage of extended-spectrum beta-lactamase-producing Enterobacteriaceae and the risk of surgical site infection after colorectal surgery: A prospective cohort study. *Clin Infect Dis* 2018 [PMID: 30204851 DOI: 10.1093/cid/ciy768]

38 **Brook I**, Walker RI, MacVittie TJ. Effect of antimicrobial therapy on bowel flora and bacterial infection in irradiated mice. *Int J Radiat Biol Relat Stud Phys Chem Med* 1988; **53**: 709-716 [PMID: 3283066 DOI: 10.1080/09553008814551081]

39 **Olivas AD**, Shogan BD, Valuckaite V, Zaborin A, Belogortseva N, Musch M, Meyer F, Trimble WL, An G, Gilbert J, Zaborina O, Alverdy JC. Intestinal tissues induce an SNP mutation in Pseudomonas aeruginosa that enhances its virulence: Possible role in anastomotic leak. *PLoS One* 2012; **7**: e44326 [PMID: 22952955 DOI: 10.1371/journal.pone.0044326]

40 **Juo YY**, Hyder O, Haider AH, Camp M, Lidor A, Ahuja N. Is minimally invasive colon resection better than traditional approaches? First comprehensive national examination with propensity score matching. *JAMA Surg* 2014; **149**: 177-184 [PMID: 24352653 DOI: 10.1001/jamasurg.2013.3660]

41 **Solaini L**, Bazzocchi F, Cavaliere D, Avanzolini A, Cucchetti A, Ercolani G. Robotic versus laparoscopic right colectomy: An updated systematic review and meta-analysis. *Surg Endosc* 2018; **32**: 1104-1110 [PMID: 29218671 DOI: 10.1007/s00464-017-5980-4]

42 **Abraha I**, Binda GA, Montedori A, Arezzo A, Cirocchi R. Laparoscopic versus open resection for sigmoid diverticulitis. *Cochrane Database Syst Rev* 2017; **11**: CD009277 [PMID: 29178125 DOI: 10.1002/14651858.CD009277.pub2]

43 **Collinson FJ**, Jayne DG, Pigazzi A, Tsang C, Barrie JM, Edlin R, Garbett C, Guillou P, Holloway I, Howard H, Marshall H, McCabe C, Pavitt S, Quirke P, Rivers CS, Brown JM. An international, multicentre, prospective, randomised, controlled, unblinded, parallel-group trial of robotic-assisted versus standard laparoscopic surgery for the curative treatment of rectal cancer. *Int J Colorectal Dis* 2012; **27**: 233-241 [PMID: 21912876 DOI: 10.1007/s00384-011-1313-6]

44 **Neutzling CB**, Lustosa SA, Proenca IM, da Silva EM, Matos D. Stapled versus handsewn methods for colorectal anastomosis surgery. *Cochrane Database Syst Rev* 2012; CD003144 [PMID: 22336786 DOI: 10.1002/14651858.CD003144.pub2]

45 **Naumann DN**, Bhangu A, Kelly M, Bowley DM. Stapled versus handsewn intestinal anastomosis in emergency laparotomy: A systemic review and meta-analysis. *Surgery* 2015; **157**: 609-618 [PMID: 25731781 DOI: 10.1016/j.surg.2014.09.030]

46 **Mann B**, Kleinschmidt S, Stremmel W. Prospective study of hand-sutured anastomosis after colorectal resection. *Br J Surg* 1996; **83**: 29-31 [PMID: 8653354 DOI: 10.1002/bjs.1800830108]

47 **ESCP Cohort Studies and Audits Committee**. The 2017 European Society of Coloproctology (ESCP) international snapshot audit of left colon, sigmoid and rectal resections - study protocol. *Colorectal Dis* 2018; **20** Suppl 6: 5-12 [PMID: 30255639 DOI: 10.1111/codi.14377]

48 **Braunschmid T**, Hartig N, Baumann L, Dauser B, Herbst F. Influence of multiple stapler firings used for rectal division on colorectal anastomotic leak rate. *Surg Endosc* 2017; **31**: 5318-5326 [PMID: 28634627 DOI: 10.1007/s00464-017-5611-0]

49 **Lee S**, Ahn B, Lee S. The Relationship Between the Number of Intersections of Staple Lines and Anastomotic Leakage After the Use of a Double Stapling Technique in Laparoscopic Colorectal Surgery. *Surg Laparosc Endosc Percutan Tech* 2017; **27**: 273-281 [PMID: 28614172 DOI: 10.1097/SLE.0000000000000422]

50 **Kim HJ**, Choi GS, Park JS, Park SY. Comparison of intracorporeal single-stapled and double-stapled anastomosis in laparoscopic low anterior resection for rectal cancer: A case-control study. *Int J Colorectal Dis* 2013; **28**: 149-156 [PMID: 23014975 DOI: 10.1007/s00384-012-1582-8]

51 **Berho M**, Wexner SD, Botero-Anug AM, Pelled D, Fleshman JW. Histopathologic advantages of compression ring anastomosis healing as compared with stapled anastomosis in a porcine model: A blinded comparative study. *Dis Colon Rectum* 2014; **57**: 506-513 [PMID: 24608308 DOI: 10.1097/DCR.0000000000000009]

52 **Masoomi H**, Luo R, Mills S, Carmichael JC, Senagore AJ, Stamos MJ. Compression anastomosis ring device in colorectal anastomosis: A review of 1,180 patients. *Am J Surg* 2013; **205**: 447-451 [PMID: 23290352 DOI: 10.1016/j.amjsurg.2012.03.013]

53 **D'Hoore A**, Albert MR, Cohen SM, Herbst F, Matter I, Van Der Speeten K, Dominguez J, Rutten H, Muldoon JP, Bardakcioglu O, Senagore AJ, Ruppert R, Mills S, Stamos MJ, Påhlman L, Choman E, Wexner SD; COMPRES collaborative study group. COMPRES: A prospective postmarketing evaluation of the compression anastomosis ring CAR 27(™) /ColonRing(™). *Colorectal Dis* 2015; **17**: 522-529 [PMID: 25537083 DOI: 10.1111/codi.12884]

54 **Slesser AA**, Pellino G, Shariq O, Cocker D, Kontovounisios C, Rasheed S, Tekkis PP. Compression versus hand-sewn and stapled anastomosis in colorectal surgery: A systematic review and meta-analysis of randomized controlled trials. *Tech Coloproctol* 2016; **20**: 667-676 [PMID: 27554096 DOI: 10.1007/s10151-016-1521-8]

55 **Ris F**, Yeung T, Hompes R, Mortensen NJ. Enhanced Reality and Intraoperative Imaging in Colorectal Surgery. *Clin Colon Rectal Surg* 2015; **28**: 158-164 [PMID: 26491408 DOI: 10.1055/s-0035-1555007]

56 **Allaix ME**, Lena A, Degiuli M, Arezzo A, Passera R, Mistrangelo M, Morino M. Intraoperative air leak test reduces the rate of postoperative anastomotic leak: Analysis of 777 laparoscopic left-sided colon resections. *Surg Endosc* 2019; **33**: 1592-1599 [PMID: 30203203 DOI: 10.1007/s00464-018-6421-8]

57 **Wu Z**, van de Haar RC, Sparreboom CL, Boersema GS, Li Z, Ji J, Jeekel J, Lange JF. Is the intraoperative air leak test effective in the prevention of colorectal anastomotic leakage? A systematic review and meta-analysis. *Int J Colorectal Dis* 2016; **31**: 1409-1417 [PMID: 27294661 DOI: 10.1007/s00384-016-2616-4]

58 **Li VK**, Wexner SD, Pulido N, Wang H, Jin HY, Weiss EG, Nogeuras JJ, Sands DR. Use of routine intraoperative endoscopy in elective laparoscopic colorectal surgery: Can it further avoid anastomotic

failure? *Surg Endosc* 2009; **23**: 2459-2465 [PMID: 19301071 DOI: 10.1007/s00464-009-0416-4]
59 **Hirst NA**, Tiernan JP, Millner PA, Jayne DG. Systematic review of methods to predict and detect anastomotic leakage in colorectal surgery. *Colorectal Dis* 2014; **16**: 95-109 [PMID: 23992097 DOI: 10.1111/codi.12411]
60 **Vignali A**, Gianotti L, Braga M, Radaelli G, Malvezzi L, Di Carlo V. Altered microperfusion at the rectal stump is predictive for rectal anastomotic leak. *Dis Colon Rectum* 2000; **43**: 76-82 [PMID: 10813128 DOI: 10.1007/BF02237248]
61 **GOLIGHER JC**. The blood-supply to the sigmoid colon and rectum with reference to the technique of rectal resection with restoration of continuity. *Br J Surg* 1949; **37**: 157-162 [PMID: 15395084 DOI: 10.1002/bjs.18003714604]
62 **Novell JR**, Lewis AA. Peroperative observation of marginal artery bleeding: a predictor of anastomotic leakage. *Br J Surg* 1990; **77**: 137-138 [PMID: 2317669 DOI: 10.1002/bjs.1800770636]
63 **Ambrosetti P**, Robert J, Mathey P, Rohner A. Left-sided colon and colorectal anastomoses: Doppler ultrasound as an aid to assess bowel vascularization. A prospective evaluation of 200 consecutive elective cases. *Int J Colorectal Dis* 1994; **9**: 211-214 [PMID: 7876727 DOI: 10.1007/BF00292253]
64 **Karliczek A**, Benaron DA, Baas PC, Zeebregts CJ, Wiggers T, van Dam GM. Intraoperative assessment of microperfusion with visible light spectroscopy for prediction of anastomotic leakage in colorectal anastomoses. *Colorectal Dis* 2010; **12**: 1018-1025 [PMID: 19681979 DOI: 10.1111/j.1463-1318.2009.01944.x]
65 **Jafari MD**, Lee KH, Halabi WJ, Mills SD, Carmichael JC, Stamos MJ, Pigazzi A. The use of indocyanine green fluorescence to assess anastomotic perfusion during robotic assisted laparoscopic rectal surgery. *Surg Endosc* 2013; **27**: 3003-3008 [PMID: 23404152 DOI: 10.1007/s00464-013-2832-8]
66 **Ris F**, Liot E, Buchs NC, Kraus R, Ismael G, Belfontali V, Douissard J, Cunningham C, Lindsey I, Guy R, Jones O, George B, Morel P, Mortensen NJ, Hompes R, Cahill RA, Near-Infrared Anastomotic Perfusion Assessment Network VOIR. Multicentre phase II trial of near-infrared imaging in elective colorectal surgery. *Br J Surg* 2018; **105**: 1359-1367 [PMID: 29663330 DOI: 10.1002/bjs.10844]
67 **Jafari MD**, Wexner SD, Martz JE, McLemore EC, Margolin DA, Sherwinter DA, Lee SW, Senagore AJ, Phelan MJ, Stamos MJ. Perfusion assessment in laparoscopic left-sided/anterior resection (PILLAR II): A multi-institutional study. *J Am Coll Surg* 2015; **220**: 82-92.e1 [PMID: 25451666 DOI: 10.1016/j.jamcollsurg.2014.09.015]
68 **Blanco-Colino R**, Espin-Basany E. Intraoperative use of ICG fluorescence imaging to reduce the risk of anastomotic leakage in colorectal surgery: A systematic review and meta-analysis. *Tech Coloproctol* 2018; **22**: 15-23 [PMID: 29230591 DOI: 10.1007/s10151-017-1731-8]
69 **Ishii M**, Tanaka E, Imaizumi T, Sugio Y, Sekka T, Tanaka M, Yasuda M, Fukuyama N, Shinozaki Y, Hyodo K, Tanioka K, Mochizuki R, Kawai T, Mori H, Makuuchi H. Local VEGF administration enhances healing of colonic anastomoses in a rabbit model. *Eur Surg Res* 2009; **42**: 249-257 [PMID: 19346745 DOI: 10.1159/000210671]
70 **Lindgren R**, Hallböök O, Rutegård J, Sjödahl R, Matthiessen P. What is the risk for a permanent stoma after low anterior resection of the rectum for cancer? A six-year follow-up of a multicenter trial. *Dis Colon Rectum* 2011; **54**: 41-47 [PMID: 21160312 DOI: 10.1007/DCR.0b013e3181fd2948]
71 **Montedori A**, Cirocchi R, Farinella E, Sciannameo F, Abraha I. Covering ileo- or colostomy in anterior resection for rectal carcinoma. *Cochrane Database Syst Rev* 2010; CD006878 [PMID: 20464746 DOI: 10.1002/14651858.CD006878.pub2]
72 **Nurkin S**, Kakarla VR, Ruiz DE, Cance WG, Tiszenkel HI. The role of faecal diversion in low rectal cancer: A review of 1791 patients having rectal resection with anastomosis for cancer, with and without a proximal stoma. *Colorectal Dis* 2013; **15**: e309-e316 [PMID: 23586660 DOI: 10.1111/codi.12248]
73 **Wu SW**, Ma CC, Yang Y. Role of protective stoma in low anterior resection for rectal cancer: A meta-analysis. *World J Gastroenterol* 2014; **20**: 18031-18037 [PMID: 25548503 DOI: 10.3748/wjg.v20.i47.18031]
74 **Hüser N**, Michalski CW, Erkan M, Schuster T, Rosenberg R, Kleeff J, Friess H. Systematic review and meta-analysis of the role of defunctioning stoma in low rectal cancer surgery. *Ann Surg* 2008; **248**: 52-60 [PMID: 18580207 DOI: 10.1097/SLA.0b013e318176bf65]
75 **Denost Q**, Rouanet P, Faucheron JL, Panis Y, Meunier B, Cotte E, Meurette G, Kirzin S, Sabbagh C, Loriau J, Benoist S, Mariette C, Sielezneff I, Lelong B, Mauvais F, Romain B, Barussaud ML, Germain C, Picat MQ, Rullier E, Laurent C; French Research Group of Rectal Cancer Surgery (GRECCAR). To Drain or Not to Drain Infraperitoneal Anastomosis After Rectal Excision for Cancer: The GRECCAR 5 Randomized Trial. *Ann Surg* 2017; **265**: 474-480 [PMID: 27631776 DOI: 10.1097/SLA.0000000000001991]
76 **Guerra F**, Giuliani G, Coletta D, Boni M, Rondelli F, Bianchi PP, Coratti A. A Meta-Analysis of Randomized Controlled Trials on the Use of Suction Drains Following Rectal Surgery. *Dig Surg* 2018; **35**: 482-490 [PMID: 29232658 DOI: 10.1159/000485139]
77 **Jesus EC**, Karliczek A, Matos D, Castro AA, Atallah AN. Prophylactic anastomotic drainage for colorectal surgery. *Cochrane Database Syst Rev* 2004; CD002100 [PMID: 15495028 DOI: 10.1002/14651858.CD002100.pub2]
78 **Yang Y**, Shu Y, Su F, Xia L, Duan B, Wu X. Prophylactic transanal decompression tube versus non-prophylactic transanal decompression tube for anastomotic leakage prevention in low anterior resection for rectal cancer: A meta-analysis. *Surg Endosc* 2017; **31**: 1513-1523 [PMID: 27620910 DOI: 10.1007/s00464-016-5193-2]
79 **Chen H**, Cai HK, Tang YH. An updated meta-analysis of transanal drainage tube for prevention of anastomotic leak in anterior resection for rectal cancer. *Surg Oncol* 2018; **27**: 333-340 [PMID: 30217286 DOI: 10.1016/j.suronc.2018.05.018]

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REVIEW

Diagnostic and prognostic potential of tissue and circulating long non-coding RNAs in colorectal tumors

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Abstract

Long non-coding RNAs (lncRNAs) are members of the non-protein coding RNA family longer than 200 nucleotides. They participate in the regulation of gene and protein expression influencing apoptosis, cell proliferation and immune responses, thereby playing a critical role in the development and progression of various cancers, including colorectal cancer (CRC). As CRC is one of the most frequently diagnosed malignancies worldwide with high mortality, its screening and early detection are crucial, so the identification of disease-specific biomarkers is necessary. LncRNAs are promising candidates as they are involved in carcinogenesis, and certain lncRNAs (*e.g.*, CCAT1, CRNDE, CRCAL1-4) show altered expression in adenomas, making them potential early diagnostic markers. In addition to being useful as tissue-specific markers, analysis of circulating lncRNAs (*e.g.*, CCAT1, CCAT2, BLACAT1, CRNDE, NEAT1, UCA1) in peripheral blood offers the possibility to establish minimally invasive, liquid biopsy-based diagnostic tests. This review article aims to describe the origin, structure, and functions of lncRNAs and to discuss their contribution to CRC development. Moreover, our purpose is to summarise lncRNAs showing altered expression levels during tumor formation in both colon tissue and plasma/serum samples and to demonstrate their clinical implications as diagnostic or prognostic biomarkers for CRC.

Key words: Long non-coding RNA; Colorectal cancer; Colorectal adenoma; Circulating long non-coding RNAs; Exosome; Biomarker; Diagnostic marker; Prognostic marker

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Core tip: The present review aims to shed light on the complex world of long non-coding RNAs (lncRNAs) by discussing their origin, localization, and functions. By summarizing the constantly growing body of knowledge about lncRNA expression in colorectal tissue and by focusing on potential circulating lncRNA markers, we aim to enhance the understanding of the comprehensive picture of their diagnostic and prognostic potential in precancerous colorectal adenomas and cancer.

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INTRODUCTION

Colorectal cancer (CRC) is one of the most frequent malignant diseases worldwide with a remarkably high mortality rate[1]. The number of CRC-related deaths can be reduced only by diagnosis at the earliest stage when the disease is more likely to be cured.

Long non-coding RNAs (lncRNAs), a novel family of non-protein coding RNAs (200 nt-10 kb) are of outstanding interest as their expression is often altered in various disease types including malignancies[2]. They are known to have a crucial role in the regulation of gene expression, alternative splicing mechanisms, protein localization and activity, formation of cellular substructures and protein complexes through their diverse interactions with DNA, RNA and proteins[3,4].

In cancers, lncRNAs are involved in every stage of carcinogenesis and tumor progression including tumor initiation, proliferation, apoptosis and migration of cancer cells, angiogenesis, tumor invasion and metastasis formation[5,6]. Their altered expression can influence several oncogenic signaling cascades including the WNT/β-catenin, PI3K/Akt, EGFR, NOTCH, mTOR and TP53 signaling pathways[4,7-20]. Besides local expression changes in cancerous tissue and tumor-related stroma, lncRNAs also remain stable in body fluids due to their resistance to RNases[2,21].

Several lncRNAs showing altered expression in colorectal tumors including precancerous adenomas have potential as early diagnostic markers[22-24]. In this review, we summarize the colorectal tumor-related tissue and circulating lncRNAs, altered lncRNA expression patterns, and technical aspects of their isolation and detection. Our aim is to show their potential as diagnostic and prognostic biomarkers based on recently published data.

HISTORY, CLASSIFICATION, FUNCTION, LIFETIME AND SUBCELLULAR LOCALIZATION OF LNCRNAS

Regulatory non-coding RNAs (ncRNAs) were first reported in eukaryotes in the 1980s, of which H19[25] and Xist[26] were the first members of the family[27]. When the Human Genome Project was completed, it became clear that only a minor part of our genome codes proteins and the rest was considered as "junk" DNA[28]. Since then, our knowledge about the non-coding genome was expanded, and the still unexplored regulatory role of the ncRNA world is the focus of several studies and holds a significant clinical potential[29]. Over the past decades, along with the development of explorative molecular biology methods, the importance and function of the complex eukaryotic transcriptome have been recognized, a large proportion of which comprises the actively transcribed lncRNAs[30]. After the discovery and the intensive analysis of the class of small ncRNAs called miRNAs since 1993[31], it became evident, that other ncRNAs also play fundamental role in gene expression regulation, and that their alterations can be responsible for the disrupted molecular pathways in multiple cancers[32].

The major class of ncRNAs are lncRNAs, which are derived from highly diverse genomic context and are classified on the basis of the genomic region of origin[28]. According to the genomic database [Ensembl Release 96 (April 2019)], human lncRNAs are categorized into 3prime overlapping ncRNA, antisense, lincRNA (long

interspersed ncRNA), retained intron, sense intronic, sense overlapping and macro lncRNAs. The lncRNAs that are not overlapping with protein-coding genes are called stand-alone lncRNAs including the large intergenic (or intervening) lncRNA (lincRNA) group[28,33] (*e.g.*, XIST, H19, MALAT1, and HOTAIR). Antisense lncRNAs are transcripts overlapping the genomic strand of a protein-coding locus in an antisense direction[34], while sense lncRNAs are overlapped with the sense strand of protein coding genes containing exons[35]. Antisense transcription is widespread in the mammalian genome[36]; the estimated ratio of the genes with antisense transcripts varies from less than 2 to more than 70% of the total genes [37]. XIST/TSIX is a well-known example of the sense-antisense transcript pairs[38]. Pseudogenes are defined as nonfunctional sequences of genomic DNA originally derived from functional genes[39]. Long intronic ncRNAs are transcribed from the intronic sequence of a coding gene. On the basis of their association with functional DNA elements, enhancer- and promoter-associated lncRNAs can be distinguished[40]. The lncRNAs localize in the cytoplasm, nucleus, nucleolus, and also in other subcellular compartments and vesicles (such as nuclear bodies, exosomes) and the localization is related to their molecular functions[41]. Certain sequence motifs in their primary sequence are associated with the subcellular localization[42].

As the largest class of non-coding transcripts, lncRNAs have a wide variety of functions. They can act as RNAs (*e.g.*, ribozymes, riboswitches)[43] and widely as ribonucleoprotein particles (RNP)[44]. They can exert their positive or negative regulating functions either *in cis* or *in trans*[45]. One of their functions is the regulation of nuclear organization; lncRNAs can modulate the chromatin architecture (*e.g.*, Xist) and they can also regulate inter- and intrachromosomal interactions (*e.g.*, colorectal cancer associated transcript 1. long isoform (CCAT1-L) modulating interchromatin loops between enhancers and promoters[46]). LncRNAs can regulate other non-coding RNAs (*e.g.*, as miRNA sponges leading to reduced miRNA inhibitory effect on target molecules[47]), and also can be processed into single- or double-stranded siRNAs[3]. Several gene transcription processes can be activated or blocked by lncRNAs by recruiting or inhibiting transcription factors of the target gene promoters[3,44]. Certain lncRNAs are linked to the process of alternative splicing (*e.g.*, LINC001133)[48]. Furthermore, protein activity is regulated by lncRNAs and trafficking between the subcellular compartments can also be influenced by lncRNAs[49].

Nuclear lncRNAs also contribute to chromatin remodeling as they can promote or prevent the recruitment of chromatin modifiers[46]. They are also part of nuclear bodies[50] with scaffold function, so-called architectural lncRNAs[51] [such as nuclear enriched abundant transcript 1 (NEAT1), as a well-characterized lncRNA as a crucial component of paraspeckles[52]] and also as non-architectural lncRNAs [*e.g.*, metastasis associated lung carcinoma transcript 1 (MALAT1) as one of the most abundant lncRNA in nuclear speckles[46]].

Epigenetic mechanisms, such as histone modifications are also influenced by lncRNAs. For instance, lncRNA HOTAIR (homeobox transcript antisense intergenic RNA) interacts with both LSD1/CoREST/REST complex and PRC2 as a modular scaffold that leads to coupled histone H3 lysine 27 methylation and lysine 4 demethylation[53].

By the modulation of all three major mammalian DNA methyltransferases (DNMT1, DNMT3a, DNMT3b), lncRNAs influence DNA methylation levels resulting in altered expression of the target genes[44]. DNMT1-associated Colon Cancer Repressed lncRNA 1 (DACOR1) interacts with both chromatin and DNMT1 and targets DNMT1 protein complex to certain genomic loci, also affecting cellular SAM levels[54,55]. Altogether, the expression alterations of lncRNAs influence many biological functions that contribute to the disturbance of the complex fine-tuning machinery of non-coding RNA regulatory network during cancer formation.

Our knowledge about the posttranscriptional regulation of lncRNAs is limited, however, the stability of transcripts can be an important aspect in gene expression regulation[56,57] as the half-life of ncRNAs correlates with their functional characteristics[58]. Each lncRNA has a unique structure, and these transcripts are characterized by complex secondary and tertiary structures which is crucial to exert their functions[59]. Although the stability of these non-coding transcripts was generally considered to be lower compared to mRNAs[60] on the basis of a genome-wide lncRNA analysis by Clark *et al*[56], a wide variety in their stability can be observed which is consistent with their functional diversity. LncRNA stability is correlated with genomic location, subcellular localization, splicing, and GC percentage, while in contrast, expression levels are not correlated with stability[56]. The half-life of lncRNAs ranges from < 30 min to > 48 h with median value at 3.5 h, and they can be classified as unstable and to highly stable lncRNAs - the latter represented at a lower percentage[56]. According to Clark *et al*., nuclear-enriched lncRNAs displayed significantly lower stability compared to those detected both in nucleus and cytoplasm[56]. It is

important to note that lncRNAs with even lower stability have been shown to have fundamental role (*e.g.*, NEAT1 as scaffold lncRNA of paraspeckles, as dynamic nuclear subdomains[61]), furthermore, the existence of highly stable lncRNAs illustrate the biomarker potential of this subclass of non-coding transcripts.

LNCRNA EXPRESSION ANALYSIS METHODS

Analysis of lncRNAs is technically challenging due to their relatively low expression level and their tissue-specific expression[62], therefore, the following methods are optimized for studying lncRNAs with high sensitivity and resolution.

High-throughput sequencing serial analysis of gene expression (SAGE) is based on short cDNA sequences containing recognition sites for restriction enzymes at the transcripts' 3' end, and it was one of the first transcriptome analysis methods to study lncRNA expression[63,64].

Among whole genome analyses, microarrays are widely used to analyse the RNA expression in a high-throughput manner from the 2000s, however, these systems are limited to studying the known RNAs. Furthermore, cross-hybridization and limited detection range due to background and saturation signals make these analyses more challenging[65]. In parallel, the rapid development of next generation sequencing (NGS) systems revolutionized the experimental field, as RNA-Seq provides a cost-effective and rapid solution for whole transcriptome profiling with the potential to discover novel transcripts[65]. The higher resolution and reproducibility of RNA-Seq compared to microarrays[65] resulted in broad use of this approach. RNA-Seq supports the annotation of novel lncRNAs, RNA editing sites, and alternative splicing sites, as well[62]. Cap analysis of gene expression (CAGE) is an NGS-based approach to map and quantify the expression of 5' capped RNAs[66] and also to identify transcriptionally active promoter regions and Pol II-driven TSSs[64].

The lncRNAs regulate and mediate interactions on different molecular levels and complex networks of these non-coding RNAs remain to be explored. RNA-binding protein immunoprecipitation (RIP) is used to study RNA-protein interactions, where the RNA of interest can be complexed with its interacting proteins, and this fraction can be selectively pulled down[67]. The downstream analysis can be performed by combining with the previously discussed methods, including RIP-Chip and RIP-Seq[68]. Native RIP is suitable for the exploration of strong and direct RNA-protein interactions, whereas the crosslinked immunoprecipitation method (CLIP) is used to study weak or indirect binding[62]. Crosslinking is achieved by ultraviolet light (UV) followed by RNase treatment and stringent washes which increases the specificity of the interaction detection[69]. In order to minimize the disadvantages of CLIP, modified methods, such as individual nucleotide resolution CLIP (iCLIP)[70], and photoactivable ribonucleoside-enhanced CLIP (PAR-CLIP) are also available for the identification of the exact crosslinking sites with single nucleotide resolution[62,69].

Other RNA pull-down methods, such as chromatin isolation by RNA purification (ChIRP)[71], capture hybridization analysis of RNA targets (CHART)[72] and RNA antisense purification (RAP)[73] can be applied to study RNA-DNA interactions to shed light on lncRNAs' functions and identify trans-genomic interacting sites[62]. During ChIRP experiments, a biotin-labeled antisense probe designed to the selected lncRNA is employed to explore its interacting chromosomal fragments[71]. Different probe design criteria are applied in the case of CHART, as in contrast with ChIRP probes spanning the whole interesting lncRNA, the CHART method uses capture oligos specific for the accessible regions of the lncRNA candidate[72]. The co-purified RNA, DNA or proteins potentially interacting with the selected lncRNA can be analysed with NGS, PCR or Western blotting[62]. RAP can be performed with different crosslinking methods (*e.g.*, psoralens) along with the longer biotinylated probes (> 60 bp) to enhance the RNA-DNA hybrid stability[73] and to reduce the signal-to-noise ratio[64].

LncRNAs are known to exert their function also by binding directly or indirectly to other RNAs[64]. These interactions can be studied by RAP-RNA (applying different chemical cross-linking), as 4'aminomethyltrioxalen: RAP-RNA[AMT], formaldehyde: RAP-RNA[FA], FA and disuccinimidyl glutarate: RAP-RNA[FA-DSG][74] or UV-crosslinked CLASH (cross-linking, ligation and sequencing of hybrids)[75] methods.

It is known that lncRNAs fold into secondary and tertiary structures that are crucial to exert their regulatory effects[59], but the structural domains of the RNA interactome still need to be explored. Structural relationships can be studied by dimethyl sulfate sequencing (DMS-Seq), selective 2'-hydroxyl acylation analysed by primer extension sequencing (SHAPE-Seq), genome-wide fragmentation sequencing (FRAG-Seq), and parallel analysis of RNA structure (PARS) techniques[76]. By the intensive development

of subcellular visualization approaches, lncRNAs can be localized within the cell with high sensitivity using special fluorescent *in situ* hybridization (FISH) applications (single molecule FISH - smFISH, sequential FISH - seqFISH, and multiplexed error-robust FISH - MerFISH)[77-79]. High resolution microscopes, as structured illumination microscopy (SIM[80]) or stochastic optical reconstruction microscopy (STORM[81]) enable the precise detection of certain lncRNAs and investigation of their colocalization partners[46]. The functional investigations of lncRNAs can be performed with antisense oligonucleotides (ASO) and also by siRNAs and shRNAs *via* binding and affecting the target lncRNA's functionality[82]. The CRISPR-Cas9 genome editing technique[83] has revolutionized functional studies in the lncRNA world, which can be employed to silence (CRISPRi[84]) and also to overexpress (CRISPRa[85]) the lncRNA of interest[86].

LNCRNA EXPRESSION ALTERATIONS IN COLORECTAL ADENOMA AND CANCER TISSUE

Increasing evidence suggest that lncRNAs are involved in the whole process of CRC development, progression and metastasis formation - similarly to their diverse regulatory role in other types of malignancies - affecting the essential signaling pathways including WNT, TP53, PI3K/Akt, mTOR, EGFR and NOTCH1 in CRC[4-20]. Abnormal expression of numerous lncRNAs including the well-known HOTAIR[87-90], MALAT1[91-93] and H19[94,95] has been described in CRC compared to normal colonic tissue samples (Table 1). From a clinical point of view, lncRNAs - with altered expression in different stages of colorectal carcinogenesis, and disease progression - have a particularly great potential to become early diagnostic and/or prognostic biomarkers.

Several studies reported the altered expression of certain lncRNAs including colon cancer associated transcript-1 (CCAT1), colorectal neoplasia differentially expressed (CRNDE-L), colorectal cancer associated lncRNA (CRCAL) 1, -2, -3 and -4 and urothelial carcinoma-associated 1 (UCA1) already in precancerous adenomas[23,24,37,96-102].

Nissan *et al*[96] in their comprehensive RT-qPCR study were the first to demonstrate the massive (often more than 100-fold) upregulation of CCAT1 in CRC and premalignant adenoma tissue samples compared to normal colonic mucosa. Furthermore, elevated CCAT1 levels could be detected in lymph node and distant liver metastases, as well as in peripheral blood mononuclear cells (PBMCs) of CRC patients[96]. Alaiyan *et al*[23] have confirmed the overexpression of CCAT1 in precancerous conditions and through all CRC stages using RT-qPCR and *in situ* hybridization (ISH). These data suggest its essential role in both early carcinogenesis and metastatic processes, moreover, *in vitro* studies revealed that the c-Myc oncogene could facilitate the transcription of CCAT1 by binding to its promoter[97]. CRNDE also becomes activated already in the initial steps of tumor development as its elevated expression was observed in > 90% of neoplastic colon tissue including adenoma and adenocarcinoma samples using both microarray and RT-PCR technology[24]. Liu *et al*[103] found significant upregulation of CRNDE-h splice variant both in adenoma and CRC tissues compared to control groups containing normal adjacent, inflammatory bowel disease and hyperplastic polyp samples. Moreover, within the CRC group, increased expression of CRNDE-h showed significant correlation with tumor size, lymph node, and distant metastasis. It was observed in *in vitro* studies, that lncRNA CRNDE can promote CRC development and progression through epigenetic silencing of dual-specificity phosphatase 5 (DUSP5) and cyclin-dependent kinase inhibitor 1A (CDKN1A)[104] or *via* activating Ras/MAPK[105] and WNT/β-catenin[106,107] signaling pathways. Furthermore, it can contribute to chemoresistance by sponging microRNAs (miR-136[108], miR-181a-5p[107]) in CRC.

Some colorectal cancer associated lncRNAs [CRCALs: CRCAL-1 (AC021218.2), CRCAL-2 (LINC00858), CRCAL-3 (RP11-138J23.1) and CRCAL-4 (RP11-435O5.2)] were identified as overexpressed and novel CRC biomarkers using RNA-sequencing techniques[100]. These lncRNAs may "be involved in the very early steps of the neoplastic process" as the expression levels of all four CRCALs were found to be elevated in colorectal adenoma samples, as well. RNA interference-mediated knockdown experiments and gene ontology analysis of The Cancer Genome Atlas (TCGA) dataset suggest the involvement of CRCAL-3 and CRCAL-4 in cell cycle regulation[100].

Several studies have also indicated the tumor-promoting role of UCA1 lncRNA in CRC[101,102,109]. Intensive UCA1 expression was found to be correlated with larger tumor size, depth of invasion, and a less differentiated histology[110]. Moreover, elevated

Table 1 Tissue and circulating long non-coding RNAs with altered expression in colorectal cancer

lncRNA	Tissue	Plasma/serum	Exosome	Expression in CRC	Ref.	Potentially diagnostic marker	Prognostic role
91H	X	X	X	Up	[130,138,152]	X (comb 1)	Up – poor prognosis
ADAMTS9-AS2	X			Down	[145]		Down – poor prognosis
AFAP1-AS1	X			Up	[145,153,154]		Up - poor prognosis
AK027294	X			Up	[155]		
AK123657/BX64820	X			Down	[156]		Down – poor prognosis
AK307796	X			Up	[157]		
ANRIL	X			Up	[158,159]		Up – poor prognosis
ATB	X	X		Up	[122,160,161]		Up - poor prognosis
BA318C17.1	X			Down	[162]		
BANCR	X	X		Down/up	[125,163,164]	X (comb 2)	Prognostic
BCAR4/HOXA-AS2	X	X	X	Up/down	[140,145]	X	Up – poor prognosis
BLACAT1	X	X		Up	[129,151,165]		Up - poor prognosis
CAHM	X			Down	[131]	X	
CASC11	X			Up	[166]		
CASC2	X			Down	[167]		Down - poor prognosis
CCAL	X			Up	[4]		Up - poor prognosis
CCAT1	X	X		Up	[23,96-98,116,168-170]	X	Up – poor prognosis
CCAT1-L	X			Up	[171]		
CCAT2	X	X		Up	[134,172,173]	X	Prognostic
CLMAT3	X			Up	[174,175]		Up – poor prognosis
CRNDE	X	X	X	Up	[24,99,103]	X	Up – poor prognosis
CRCAL-1/AC021218.2	X			Up	[100]	X	
CRCAL-2/LINC00858	X			Up	[100]	X	
CRCAL-3/RP11-138J23.1	X			Up	[100]	X	
CRCAL-4/RP11-453O5.2	X			Up	[100]	X	
CTD903	X			Down	[176]		Down - poor prognosis
CTNNAP1	X			Down	[177]	X	
DACOR1	X			Up	[54]		
DANCR	X			Up	[178]		Up – poor prognosis
DQ786243	X			Up	[179]		Prognostic
E2F4 antisense	X			Up	[180]		
ENST00000430471	X			Up	[181]		
ENST00000455974/AC123023.1	X			Up	[111]	X	Up - poor prognosis
ENST00000465846	X			Down	[157]		
FER1L4	X	X		Down	[123]		Down - poor prognosis

FEZF1-AS1	X			Up	[115,182]		Up - poor prognosis
FTX	X			Up	[183]		Up – poor prognosis
GAPLINC	X			Up	[184]		Prognostic
GAS5	X	X	X	Down	[120,185-188]		Down - poor prognosis
GHET1	X			Up	[189]		
lnc-GNAT1-1		X			[119]		
H19	X			Up	[94,143-145,190,191]		Up - poor prognosis
HIF1-AS1	X	X			[132]		Up - poor prognosis
HIF2PUT	X			Up	[192]		
HOTAIR	X	X		Up	[87-90,116,193,194]		Up - poor prognosis
HOTAIRM1	X	X		Down	[117]		Down – poor prognosis
HOTTIP	X			Up	[195,196]		Up – poor prognosis
HULC	X	X		Up	[134,197]		Up - poor prognosis
KCNQ1OT1	X			Up	[198,199]		
LINC00152 (CYTOR)	X			Up	[8,11,13]		Up – poor prognosis
LINC01133	X			Down	[200]		Down - poor prognosis
LINC01296	X			Down	[201]		Down – poor prognosis
lincRNA-p21	X	X		Down	[116,202-204]		Down – poor prognosis
Lnc34a	X			Up	[205]		
lncRNA-LET/NPNT-IT1	X			Down	[206]		
LNCV6_116109			X	Up	[139]	X	
LNCV6_98390			X	Up	[139]	X	
LNCV6_38772			X	Up	[139]	X	
LNCV6_108226			X	Up	[139]	X	
LNCV6_84003			X	Up	[139]	X	
LNCV6_98602			X	Up	[139]	X	
LOC152578		X		Up	[126]	X (comb 3)	
LOC100287225	X			Down	[207,208]		
LOC285194/TUSC7	X	X		Down/Up	[124,209,210]	X (comb 4)	Down - poor prognosis
Loc554202	X			Down	[211,212]		Down - poor prognosis
MALAT1	X	X		Up	[7,91-93,145,149,213,214]		Up - poor prognosis
MEG3	X	X		Down	[100,130,215-218]	X (comb 1)	Down - poor prognosis
Nbla12061		X		Up	[124]	X (comb 4)	
ncNRFR	X			Up	[219]		
ncRAN	X			Down	[220,221]		Down – poor prognosis
ncRuPAR	X			Down	[222]		Down – poor prognosis
NEAT1	X	X		Up	[128,223]		Up - poor prognosis
NORAD		X			[133]		
NR_026817		X		Down	[125]	X (comb 2)	Prognostic
NR_029373	X	X		Down	[125]	X (comb 2)	Down – poor prognosis

NR_034119		X		Down	[125]	X (comb 2)	
PANDAR	X			Up	[224]		Up - poor prognosis
PCAT-1	X			Up	[225]		Up – poor prognosis
PRNCR1	X			Up	[226,227]		
PVT-1	X	X		Up	[130,145,228]	X (comb 1)	Up - poor prognosis
RP1-13P20.6	X			Down	[229]		Down - poor prognosis
RP11-462C24.1	X	X		Down/Up	[124,230,231]	X (comb 4)	Down - poor prognosis
SLC25A25-AS1	X	X		Down	[118]		Down - poor prognosis
SnaR	X				[232]		
SNHG20	X			Up	[233]		Prognostic
SNHGI2	X			Up	[234]		Up - poor prognosis
Sox2ot	X			Up	[235]		
SPRY4-IT1	X	X		Up	[121,236]		Up - poor prognosis
TINCR	X			Down	[100,237]		
TUG1	X	X	X	Up	[101,238-240]		Up - poor prognosis
uc.388	X			Down/Up	[241,242]	X	Prognostic
uc.73a	X			Down/Up	[241,242]	X	
uc002kmd.1	X			Up	[243]		
UCA1	X	X	X	Up	[100,101,110,244,245]		Up - poor prognosis
UPAT	X			Up	[246]		
XIST	X	X		Up	[247,248]		Prognostic
XLOC_000303		X		Up	[136]	X (comb 3)	
XLOC_006844	X	X		Up	[136]	X (comb 3)	
ZFAS1	X	X		Up	[19,249]		Up - poor prognosis
ZNF582-AS1	X			Down	[250]	X	

The altered expression of long non-coding RNAs in colorectal cancer tissue, plasma/serum or exosomes and the potential diagnostic value are marked with X, respectively. Combined marker sets are also represented (comb 1, comb 2, comb 3 and comb 4).

UCA1 levels could be detected in precancerous adenomas which increase in CRC[102].

In a recent publication, Lao *et al*. have described the gradual elevation of expression of a novel lncRNA, AC123023.1-201 (ENST0000455974) along the colonic normal-adenoma-dysplasia-carcinoma-metastasis sequence[111]. High levels of this lncRNA were found to be significantly associated with poor survival of DNA mismatch repair proficient (pMMR) CRC patients. *In vitro* studies suggest that AC123023.1-201 might exert an oncogenic role in the pathomechanism of pMMR CRC *via* promoting JAG2-mediated Notch signaling[111].

LNCRNA MARKERS IN PLASMA/SERUM OF COLORECTAL TUMOR PATIENTS AND THEIR MALIGNANCY-RELATED CELL FUNCTIONS

LncRNA molecules can cross the cell membrane, and hence can be found in different body fluids, such as blood, plasma/serum or urine[112]. They can be derived from apoptotic and necrotic cells, or from living cells by an active manner. These molecules occur in association with RNA-binding proteins or lipoprotein complexes, however, extracellular vesicles are reported to be the primary source of plasma lncRNAs[113]. These forms contribute to the relative resistance to degradation by RNase enzymes that make circulating lncRNAs promising markers for the prognosis, diagnosis, or screening of various diseases, including CRC[114]. The altered expression levels of

several lncRNAs were reported in tumor tissues of CRC patients, and recently, additional articles have been published describing their presence in plasma or serum samples[115]. CCAT1 and HOTAIR are among the first markers reported to have significantly elevated expression in the plasma of CRC patients compared to healthy controls[116]. It was also observed that after surgical treatment of CRC patients, the serum levels of these lncRNAs decreased in comparison with pre-operative samples. HOTAIR expression was also reported in peripheral blood mononuclear cells (PBMC) of CRC blood donors as compared with controls; of note, patients with right-sided CRC had lower levels of HOTAIR lncRNA than those with left-sided cancers[90]. In contrast, HOX antisense intergenic RNA myeloid 1 (HOTAIRM1) showed reduced expression in tumor tissue, and low levels were reported in plasma of CRC patients compared to healthy controls using nested TaqMan RTPCR method[117]. It has been assumed that this lncRNA can inhibit intense cell division and therefore, it may function as a tumor suppressor. The expression of lncRNA SLC25A25-AS1 was also significantly decreased in both tumor tissue and serum samples, and based on *in vitro* measurements, it was observed that downregulation of SLC25A25-AS1 has an impact on chemoresistance and induces the epithelial-mesenchymal transition (EMT) process[118]. Low levels of lnc-GNAT1-1 were detected in the plasma of CRC patients, and with advanced TNM stages, the level of this lncRNA decreased in the peripheral blood[119]. LncRNA growth arrest specific transcript 5 (GAS5) had diminished expression in serum samples of 109 CRC patients compared with 99 healthy controls[120]. Further experiments highlighted that low level of GAS5 was correlated with advanced TNM stages and larger tumor size. LncRNAs that can enhance cell proliferation were also described in some reports. For instance, lncRNA SPRY4-IT1 was found to be significantly upregulated in CRC tissue and serum samples, and its increased expression was associated with late TNM stages. It influences proliferation, migration, and invasion of CRC cells, and has an effect on the expression of EMT-related genes[121]. Long non-coding RNA-activated by TGF-β (lncRNA-ATB) has been analysed in 50 preoperative and postoperative plasma samples of cancer patients and in 50 healthy volunteers, and its overexpression was reported in 70% (35/50) of CRC cases one month after surgery[122]. Moreover, lncRNA-ATB levels were found to be significantly higher in postoperative plasma in comparison with preoperative samples, suggesting that lncRNA might be released by other mechanisms than by the primary tumor. This research group described another lncRNA, fer-1-like protein 4 (FER1L4) that showed decreased expression level in postoperative blood samples compared with the matched preoperative ones in contrast to the above-mentioned lncRNA-ATB[123]. Wang *et al*. compiled a panel of lncRNA containing 3 RNAs (LOC285194, RP11-462C24.1, and Nbla12061) that were upregulated in 61 CRC serum samples compared to healthy controls (n = 60)[124]. Another study selected four lncRNAs (BANCR, NR_026817, NR_029373, and NR_034119) for further experiments after high-throughput microarray analysis, and concluded that this panel was dysregulated in tissue and serum samples of colon carcinoma patients[125]. Shi *et al*[126] also performed microarray analysis on the circulating plasma lncRNA fraction using Human LncRNA Array v3.0, and 8 transcripts were further examined with RT-qPCR technique. From these candidates, expression of three (XLOC_006844, LOC152578, and XLOC_000303) lncRNAs were found to be significantly higher in CRC plasma samples (n = 220) compared to cancer-free controls (n = 180). Another lncRNA, nuclear-enriched abundant transcript 1 (NEAT1) was identified based on microarray results as the most significantly upregulated gene in whole blood samples of CRC patients[127]. Two variants of this lncRNA, NEAT1_v1 and NEAT1_v2 were studied separately, and high levels of both two transcripts were observed[128]. Moreover, Wu *et al*[129] showed that knockdown of NEAT1_v1 caused inhibition of cell invasion and proliferation *in vitro*, while in case of NEAT1_v2, the knockdown of the transcript could induce cell growth. Similarly to the previous studies, lncRNA bladder cancer associated transcript 1 (BLACAT1) was also found to be overexpressed using microarray analysis and the increased expression was confirmed using RT-PCR in CRC serum samples. Liu *et al*. selected 3 lncRNAs, H19 antisense (91H), plasmocytoma variant translocation 1 (PVT-1) and maternally expressed gene 3 (MEG3) and reported increased levels in plasma of CRC patients compared to non-cancerous controls[130]. Our knowledge on the regulation of lncRNA gene expression is incomplete; however, a study by Pedersen *et al*[131] demonstrated reduced level of lncRNA CAHM in CRC patients coupled with elevated methylation of CAHM gene, which was detectable also in plasma samples.

Additional circulating lncRNAs have been described as potential biomarkers for CRC detection (*e.g*., HIF1A-AS1, NORAD, CCAT2 or HULC), and more are expected to be identified in the near future [132-134]. The most promising lncRNAs to date are summarised in Table 1.

APPEARANCE OF LONG NON-CODING RNAS IN EXOSOMES

Exosomes are a subgroup of extracellular vesicles (EVs) that can be found in different body fluids, including blood, serum/plasma, urine or saliva. The particles range from 30 to 100 nm in diameter, and around 2×10^{15} exosomes have been identified in the blood of healthy people; however, in case of cancer, the exosome numbers can increase, and reaching 4×10^{15}[135,136]. Recent studies highlighted that exosomes secreted by tumor cells contain DNAs, proteins, lipids, different small molecules and RNAs including lncRNAs, and these molecules may also be taken by target cells. Therefore, the contents of exosomes can influence the biological functions of the recipient cells and play an important part in long distance cell-cell communication[137].

Several differentially expressed lncRNAs in exosomes were reported in plasma/serum samples of CRC patients. According to Liu *et al*[103], colorectal neoplasia differentially expressed-h (CRNDE-h) showed elevated expression in isolated exosomes of 148 CRC patients compared to benign colorectal disease patients and healthy controls. Moreover, it was observed that a high exosomal level of this lncRNA correlated with both lymph node and distant metastasis and was related to low overall survival rates. Expression of exosomal lncRNA 91H also increased in CRC serum samples, which occurs at a higher level in the vesicles, than in exosome-free sera[138]. It has been also reported that the elevated expression was decreased after surgery. Based on real-time PCR results, Barbagallo *et al*[101] demonstrated that UCA1 in serum exosomes of cancerous patients was downregulated, while taurine up-regulated 1 (TUG1) was overexpressed. Another study constructed a six-member (LNCV6_116109, LNCV6_98390, LNCV6_38772, LNCV_108266, LNCV6_84003 and LNCV6_98602) panel of plasma exosomal lncRNAs based on microarray analysis that indicated overexpression in CRC patients compared to healthy individuals[139]. The increased level was already observed in the early stages of CRC suggesting that these lncRNAs are potential markers for early detection of cancer. Dong *et al*[140] showed that two mRNAs (KRTAP5-4 and MAGEA3) and one lncRNA (BCAR4) extracted from sera exosomes are present at a lower level in colorectal adenoma and carcinoma patients compared to healthy individuals, and the combination of these RNAs could be used as CRC biomarkers. Interestingly, according to Li *et al*[120] lncRNA GAS5 was found to be downregulated in CRC sera samples and acts as a tumor suppressor in cancer development, however, another study revealed that this lncRNA was upregulated in tissues, plasma and exosomes of CRC patients and its expression was related to TNM stage, Dukes stage, lymph node metastasis, local recurrence rate and distant metastasis rate[141].

Analysis of lncRNAs in exosomes is ongoing, and because altered levels of lncRNAs can serve as a potential markers for CRC detection, clarification of their function in cancer development is also a crucial step. The exosomal lncRNAs with altered expression in CRC are listed in Table 1.

CLINICAL RELEVANCE OF ALTERED LONG NON-CODING RNA EXPRESSION PATTERNS IN CRC

Biomarkers - as objectively measurable molecules suitable for monitoring physiological and pathological processes and the effect of treatments - have a crucial role in the clinical workup of tumors, enhancing the early diagnosis, classification of tumors, monitoring therapy response, and supporting the evolvement of personalized therapies, as well[21]. LncRNAs can serve as diagnostic, prognostic and predictive biomarkers in malignant diseases including CRC[22]. Principally, lncRNAs with altered levels in different stages of tumorigenesis and progression have a great potential to become early diagnostic and/or prognostic biomarkers. Besides the remarkable expression difference associated with disease stages, the important aspect of their presence and stability in the circulatory system are opening a new path for noninvasive diagnostic applications[21,142]. CCAT1 can serve as a promising marker for early CRC recognition due to its high expression in malignant and benign colorectal tumors compared to normal controls[23,96], and its detection both in PBMC and plasma samples, as well[96,116]. Increased plasma CCAT1 could predict the presence of CRC with 75.7% sensitivity and 85.3% specificity[116]. Almost all splice variants of CRNDE lncRNA, (except for CRNDE-d), and particularly CRDNME-b and CRNDE-h, were found to be intensively (approximately 5- to 100-fold) upregulated in both benign and malignant neoplastic colorectal tissue[24]. On the basis of CRNDE-h expression levels, CRC and normal tissue samples could be discriminated with 85% sensitivity and 96% specificity, which was also proven to be a highly sensitive and specific marker in

adenoma *vs* normal tissue comparison (sensitivity: 95%, specificity: 96%)[24]. Based on CRNDE-h levels in tissue, CRC could be differentiated from adenoma and healthy tissues with 70.4% sensitivity and 70.8% specificity[99]. Its strong diagnostic potential was also supported by the circulating CRNDE-h RT-qPCR results at a reported 87% sensitivity and 93% specificity between CRC *vs* healthy controls[24]. Moreover, the analysis of exosomal CRC-related CRNDE-h of serum also allowed separation of CRC samples from benign and healthy controls (AUC = 0.892, sensitivity: 70.3%, specificity: 94.4%)[103]. The newly identified upregulated CRCAL1-4 lncRNAs might be suitable for early recognition of colorectal neoplasias, however, only marginal significance could be observed between adenoma and CRC[100]. Potential utilization in CRC screening and diagnostics of several other differentially expressed lncRNAs including BLACAT1[129], CCAT2[134], HULC[134], NEAT1[128], UCA1[101,109] and HOTAIRM1[117] has also emerged in RT-qPCR studies analyzing circulating lncRNAs resulting in various specificity (43%-96%) and sensitivity (55%-100%) values. In addition to the altered expression levels, the DNA methylation changes of lncRNAs can hold a discriminative ability, as the amount of methylated CAHM DNA molecules in the circulatory system depends on the CRC stages; hence it can serve as a promising marker for CRC screening[131].

In addition to single lncRNA marker candidates, lncRNA marker combinations and multi-marker lncRNA panels have also been identified as a potential diagnostic approach. By testing the CRC diagnostic efficacy of circulating HOTAIR and CCAT1, the combined measurement of their plasma/serum levels resulted in higher sensitivity and specificity values (84.3% and 80.2%, respectively) than the above-mentioned markers alone[116]. This marker combination could provide an effective CRC diagnosis performance, moreover, it could detect CRC efficiently already at an early stage (85%). Analysis of Barbagallo *et al*[101] revealed that diagnostic accuracy of serum exosome UCA1 levels for CRC (sensitivity: 100%, specificity: 43%) could be enhanced by applying it in combination with TUG1 lncRNA (sensitivity: 93%, specificity: 64%) or with circHIPK3 circular non-coding RNA (sensitivity: 100%, specificity: 70%). A promising lncRNA panel containing three lncRNAs (LOC152578, XLOC_000303, and XLOC_0006844) upregulated in CRC was identified and validated on a large independent plasma sample cohort (220 CRCs, 180 controls) (positive predictive value: 0.80, negative predictive value: 0.84, AUC = 0.975)[126]. The double-blind test on another 100 plasma samples (50 CRC, 50 cancer-free controls) also confirmed that the above-mentioned biomarker set is suitable for indicating the occurrence of CRC with 85% accuracy[126]. CRC and healthy normal cases could be distinguished based on the increased serum levels of LOC285194, RP11-46C24.1, and Nbla12061 lncRNAs (AUC = 0.793, sensitivity: 68.33%, specificity: 86.89%)[124]. The predictive value of this lncRNA signature was significantly higher than of the conventional clinical serum protein markers (CEA, CA199, CA125, and CA724) (AUC values were 0.633, 0.567, 0.517 and 0.592, respectively)[124]. Microarray analysis of CRC-NAT tissue sample pairs revealed a four-lncRNA panel (upregulated BANCR and downregulated NR_026817, NR_029373, NR_034119) which had consistently altered pattern both in CRC tissue and serum samples compared to normal controls[125]. The high AUC, specificity and sensitivity values for both the training and validation sample sets support the reliable diagnostic ability of this biomarker set (AUC: 0.891 and 0.881; specificity: 80% and 75.83%; sensitivity: 81.67% and 89.17%) which even exceeded the diagnostic power of CEA[125]. A pilot study of Liu *et al*. revealed a new promising diagnostic plasma ncRNA biomarker set (H91, PVT-1, MEG3) for early-stage CRCs as the panel could differentiate CRC samples from controls with 82.76% sensitivity and 78.57% specificity[130].

According to the Lnc2Cancer 2.0 database (www.bio-bigdata.com/lnc2cancer), the most frequently described lncRNAs with prognostic value in CRC are H19[95,143-145], CRNDE[99,103,105,107,146], HOTAIR[89,90,147,148] and MALAT1[92,145,149] (Supplemental Table 1). *In silico* lncRNA expression analysis of CRC data from The Cancer Genome Atlas (TCGA) database (n = 534) showed that H19 was the lncRNA mostly associated with the overall survival (OS) of CRC patients (P = 0.0005), independently from tumor stages[143]. Elevated H19 levels were found to be correlated with tumor differentiation and advanced TNM stage[144], and its expression could be considered as an independent predictor for OS and disease-free survival (DFS). Other studies also confirmed that overexpression of H19 lncRNA could predict the unfavorable prognosis in CRC[145]. CRNDE-h can serve as a promising early diagnostic biomarker for CRC, and it also has a prognostic capability due to its high tissue and serum exosome levels significantly correlated with tumor size, lymph node, and distant metastasis[99,103]. In addition, increased exosomal CRNDE-h levels were proven to be a negative predictor of OS of CRC patients [34.6% (high CRNDE-h) *vs* 68.2% (low CRNDE-h), P < 0.001)][103]. Similar associations with CRC stages were reported for CRNDE-p, another overexpressed transcript variant of CRNDE[146]. HOTAIR lncRNA

was also observed to be a negative prognostic factor in CRC, as its upregulated expression in primary tumor tissue, even more in blood of CRC patients were found to be associated with higher mortality [Cox's proportional hazard, hazard ratio (HR) (tissue) = 4.4, HR (blood) = 5.9][90]. Significant differences in clinicopathological parameters such as less differentiated histology, greater tumor depth, and liver metastasis were observed in CRC cases with high HOTAIR expression (n = 20) compared CRCs with low HOTAIR levels (n = 80) ($P < 0.05$)[89]. Results of several other studies verified the correlation of higher HOTAIR levels with poorer OS[89,148]. With the RT-qPCR analysis of tissue samples from 146 stage II/III CRC patients, it was observed that patients with more intense MALAT1 lncRNA expression had a significantly worse prognosis with a HR of 2.863 for DFS and 3.968 for OS[92]. Moreover, high MALAT1 levels were found to be associated with decreased patient survival and poor response to oxaliplatin-based chemotherapy in advanced CRC patients suggesting its utility as a prognostic marker and therapeutic target in CRC[149].

In addition to CRNDE[103,146] and HOTAIR[90,116], among the 31 potentially prognostic lncRNAs published in at least two independent studies, CCAT2[150], GAS5[141], BLACAT1[129], CCAT1[96,116], NEAT1[128], 91H[138] and BANCR[125] lncRNAs were also detectable in the circulation suggesting their application as minimally invasive markers for CRC prognosis (Table 1 and Supplemental Table 1). As reported by Ozawa *et al*[150] in a study involving two independent cohorts, the evaluation of CCAT2 expression in combination with CCAT1 may be a powerful tool for predicting tumor recurrence and prognosis in CRC patients. According to the expression analysis in tissue, plasma and exosome samples, GAS5 had a prognostic value in CRC based on its expression that was negatively correlated with TNM status, Dukes stage, and lymph node metastasis (LNM), local recurrence and distant metastasis rate, while its level was in positive relation with differentiation degree and the 3-year OS rate[141]. On the other hand, elevated BLACAT1 expression could be considered as an independent unfavorable prognostic indicator for CRC, as it was observed to be associated with advanced CRC stages and shorter OS[151]. The predictive potential of lncRNA transcript variants can differ, as the OS of CRC patients with intensive NEAT1_v1 expression was worse, while high levels of the other isoform, NEAT1_v2 was correlated with better OS[128]. Determination of clinical significance of elevated exosomal H91 lncRNA expression suggested that it might be an early minimal invasive biomarker for CRC recurrence or metastasis[138]. Gong *et al*[132] evaluated the diagnostic and prognostic value of increased serum HIF1A-AS1 levels in 151 CRC and 160 healthy control samples by RT-PCR, and reported a high diagnostic efficacy (86.8% sensitivity and 92.5% specificity); moreover, it was described as a predictor for worse prognosis in CRC.

In addition to the diagnostic and prognostic utility of lncRNAs with altered expression, ongoing research focused on the role of lncRNAs in chemoresistance and therapy response prediction are revealing several lncRNAs which could be promising therapeutic targets in CRC. Similarly to the above-mentioned MALAT1 whose increased levels were found to be associated with poor response to oxaliplatin (OXA)-based chemotherapy[149], CRNDE can also contribute to oxaliplatin resistance in CRC[107,108]. According to a recent *in vitro* study, CRNDE facilitates the resistance against OXA or 5-fluorouracil (5FU) treatment *via* miR-181a-5p-mediated regulation of Wnt/β-catenin signaling[107]. Association between high HOTAIR expression and poor response to 5FU treatment was assessed[147]. HOTAIR can contribute to 5FU resistance through suppressing miR-218 and activating NF-κB signaling in CRC[147]. HOTAIR was observed to be upregulated in drug-resistant cisplatin- or paclitaxel-treated SW620 and Colo205 CRC cells, as well[148] and could affect the chemoresistance of CRC via miR-203a-3p-mediated modulation of Wnt/β-Catenin pathway[148]. The most important tissue and circulating lncRNAs with diagnostic and prognostic potential in colorectal tumors are represented in Figure 1.

CONCLUSION

The increasing number of genome-wide expression analysis studies have led to the identification of a number of long non-coding RNAs with altered expression patterns in cancers including CRC. LncRNAs are proven to contribute to each step of the colorectal carcinogenesis and tumor progression by influencing the key cancer-related signal transduction pathways such as WNT/β-catenin, PI3K/Akt, EGFR, NOTCH, mTOR and TP53 signaling. Dysregulated lncRNAs can appear in the pre-malignant adenoma stage of CRC and the expression alterations of a relatively large number of lncRNAs were found to be associated with clinicopathological parameters indicating CRC progression. Furthermore, lncRNAs are stable and detectable in body fluids

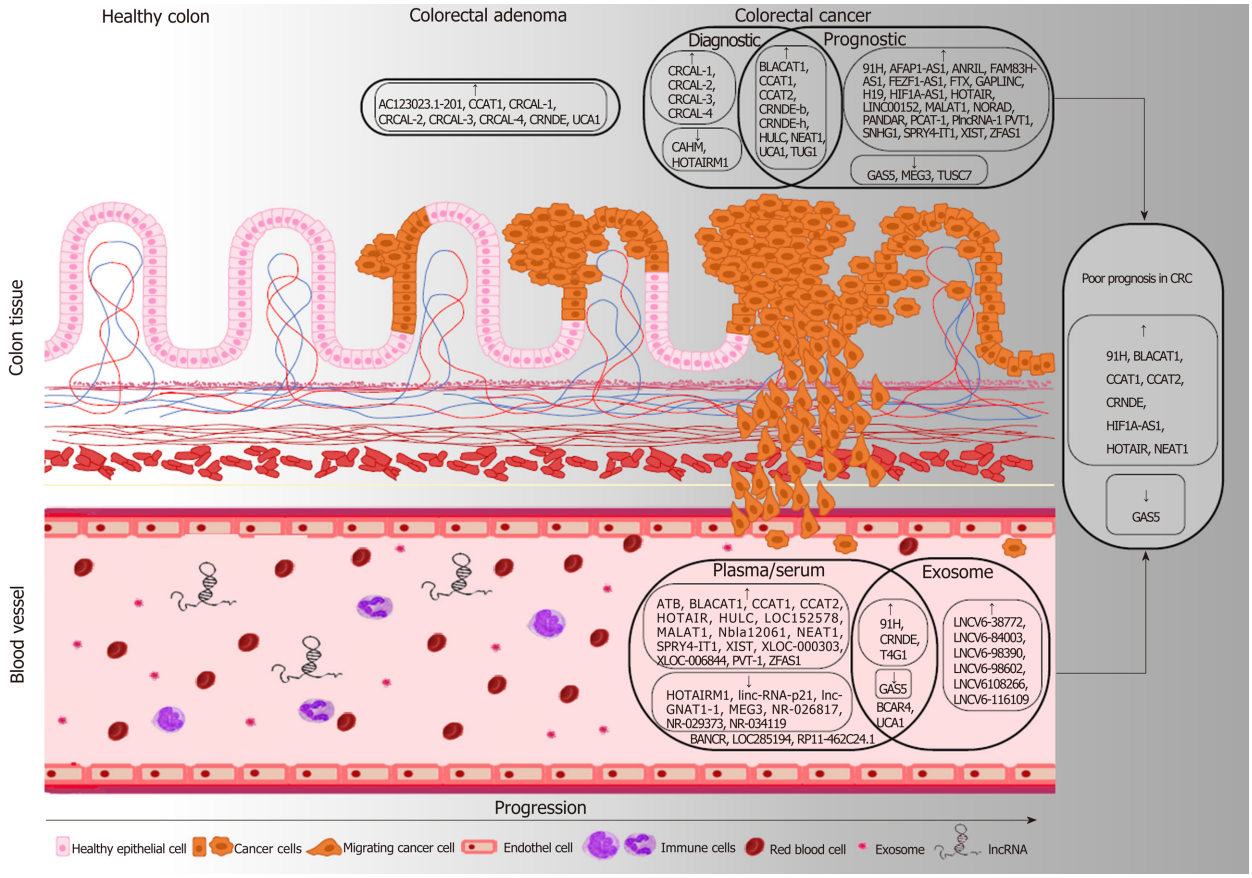

Figure 1 The most important tissue and circulating long non-coding RNA candidates with diagnostic and prognostic potential in colorectal tumors. Long non-coding RNA (lncRNAs) upregulated in adenoma or colorectal cancer (CRC) samples compared to normal controls are marked with ↑, while the downregulated lncRNAs are depicted with ↓. Potential prognostic markers detectable both in tissue and blood specimens are highlighted in the right, where ↑ refers to lncRNAs whose higher levels were found to be associated with poor prognosis (CRNDE, HOTAIR, CCAT2, BLACAT1, CCAT1, NEAT1, 91H, HIF1A-AS1), while the low expression of lncRNA marked with ↓(GAS5) can be a predictor of worse disease outcome in CRC patients. In case of the lncRNAs written without frame (BANCR, BCAR4, LOC285194, RP11-462C24.1, UCA1), diverse, sometimes controversial expression data are available in the scientific literature.

facilitating their utilization as early detection and prognostic biomarkers. In order to open the door for implementation of minimally invasive lncRNA-based tests in the clinical practice, certain relevant technical aspects should be considered: (1) Standardization of the pre-processing and sample preparation procedure including the applied blood collection tubes, sample storage conditions and time, optimized lncRNA isolation protocols from liquid biopsy samples; (2) Selection of appropriate quantification, quality checking and sensitive techniques allowing the precise detection of cancer-related alterations; and (3) Application of proper universal endogenous controls for increasing the reliability and the accuracy of RT-qPCR measurements. For the development of adequately sensitive and CRC-specific, clinically applicable diagnostic and prognostic tests based on lncRNA markers/marker panels, validation studies with large sample cohorts are essential. On the other hand, as recent studies shed light on the potential role of lncRNAs as novel therapeutic targets, the specific lncRNA expression alterations in liquid biopsy samples may contribute to the improved early recognition, prognosis prediction and therapy monitoring in CRC. Moreover, lncRNAs as druggable targets might represent the basis of novel therapeutic methods in the fight against cancer.

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REFERENCES

1 **Ferlay J**, Colombet M, Soerjomataram I, Dyba T, Randi G, Bettio M, Gavin A, Visser O, Bray F. Cancer incidence and mortality patterns in Europe: Estimates for 40 countries and 25 major cancers in 2018. *Eur J Cancer* 2018; **103**: 356-387 [PMID: 30100160 DOI: 10.1016/j.ejca.2018.07.005]

2 **Shi T**, Gao G, Cao Y. Long Noncoding RNAs as Novel Biomarkers Have a Promising Future in Cancer Diagnostics. *Dis Markers* 2016; **2016**: 9085195 [PMID: 27143813 DOI: 10.1155/2016/9085195]

3 **Gutschner T**, Diederichs S. The hallmarks of cancer: a long non-coding RNA point of view. *RNA Biol* 2012; **9**: 703-719 [PMID: 22664915 DOI: 10.4161/rna.20481]

4 **Ma Y**, Yang Y, Wang F, Moyer MP, Wei Q, Zhang P, Yang Z, Liu W, Zhang H, Chen N, Wang H, Wang H, Qin H. Long non-coding RNA CCAL regulates colorectal cancer progression by activating Wnt/β-catenin signalling pathway via suppression of activator protein 2α. *Gut* 2016; **65**: 1494-1504 [PMID: 25994219 DOI: 10.1136/gutjnl-2014-308392]

5 **Zhang H**, Chen Z, Wang X, Huang Z, He Z, Chen Y. Long non-coding RNA: a new player in cancer. *J Hematol Oncol* 2013; **6**: 37 [PMID: 23725405 DOI: 10.1186/1756-8722-6-37]

6 **Qiu MT**, Hu JW, Yin R, Xu L. Long noncoding RNA: an emerging paradigm of cancer research. *Tumour Biol* 2013; **34**: 613-620 [PMID: 23359273 DOI: 10.1007/s13277-013-0658-6]

7 **Ji Q**, Liu X, Fu X, Zhang L, Sui H, Zhou L, Sun J, Cai J, Qin J, Ren J, Li Q. Resveratrol inhibits invasion and metastasis of colorectal cancer cells via MALAT1 mediated Wnt/β-catenin signal pathway. *PLoS One* 2013; **8**: e78700 [PMID: 24244343 DOI: 10.1371/journal.pone.0078700]

8 **Yue B**, Liu C, Sun H, Liu M, Song C, Cui R, Qiu S, Zhong M. A Positive Feed-Forward Loop between LncRNA-CYTOR and Wnt/β-Catenin Signaling Promotes Metastasis of Colon Cancer. *Mol Ther* 2018; **26**: 1287-1298 [PMID: 29606502 DOI: 10.1016/j.ymthe.2018.02.024]

9 **Xu S**, Sui S, Zhang J, Bai N, Shi Q, Zhang G, Gao S, You Z, Zhan C, Liu F, Pang D. Downregulation of long noncoding RNA MALAT1 induces epithelial-to-mesenchymal transition via the PI3K-AKT pathway in breast cancer. *Int J Clin Exp Pathol* 2015; **8**: 4881-4891 [PMID: 26191181]

10 **Zhou J**, Zhi X, Wang L, Wang W, Li Z, Tang J, Wang J, Zhang Q, Xu Z. Linc00152 promotes proliferation in gastric cancer through the EGFR-dependent pathway. *J Exp Clin Cancer Res* 2015; **34**: 135 [PMID: 26538117 DOI: 10.1186/s13046-015-0250-6]

11 **Yue B**, Cai D, Liu C, Fang C, Yan D. Linc00152 Functions as a Competing Endogenous RNA to Confer Oxaliplatin Resistance and Holds Prognostic Values in Colon Cancer. *Mol Ther* 2016; **24**: 2064-2077 [PMID: 27633443 DOI: 10.1038/mt.2016.180]

12 **Cai Q**, Wang ZQ, Wang SH, Li C, Zhu ZG, Quan ZW, Zhang WJ. Upregulation of long non-coding RNA LINC00152 by SP1 contributes to gallbladder cancer cell growth and tumor metastasis via PI3K/AKT pathway. *Am J Transl Res* 2016; **8**: 4068-4081 [PMID: 27829993]

13 **Bian Z**, Zhang J, Li M, Feng Y, Yao S, Song M, Qi X, Fei B, Yin Y, Hua D, Huang Z. Correction: Long non-coding RNA LINC00152 promotes cell proliferation, metastasis, and confers 5-FU resistance in colorectal cancer by inhibiting miR-139-5p. *Oncogenesis* 2018; **7**: 63 [PMID: 30115914 DOI: 10.1038/s41389-018-0067-1]

14 **Yang X**, Duan B, Zhou X. Long non-coding RNA FOXD2-AS1 functions as a tumor promoter in colorectal cancer by regulating EMT and Notch signaling pathway. *Eur Rev Med Pharmacol Sci* 2017; **21**: 3586-3591 [PMID: 28925486]

15 **Lu S**, Dong W, Zhao P, Liu Z. lncRNA FAM83H-AS1 is associated with the prognosis of colorectal carcinoma and promotes cell proliferation by targeting the Notch signaling pathway. *Oncol Lett* 2018; **15**: 1861-1868 [PMID: 29434883 DOI: 10.3892/ol.2017.7520]

16 **Ji J**, Tang J, Deng L, Xie Y, Jiang R, Li G, Sun B. LINC00152 promotes proliferation in hepatocellular carcinoma by targeting EpCAM via the mTOR signaling pathway. *Oncotarget* 2015; **6**: 42813-42824 [PMID: 26540343 DOI: 10.18632/oncotarget.5970]

17 **Jahangiri B**, Khalaj-Kondori M, Asadollahi E, Sadeghizadeh M. Cancer-associated fibroblasts enhance cell proliferation and metastasis of colorectal cancer SW480 cells by provoking long noncoding RNA UCA1. *J Cell Commun Signal* 2019; **13**: 53-64 [PMID: 29948578 DOI: 10.1007/s12079-018-0471-5]

18 **Sun L**, Jiang C, Xu C, Xue H, Zhou H, Gu L, Liu Y, Xu Q. Down-regulation of long non-coding RNA RP11-708H21.4 is associated with poor prognosis for colorectal cancer and promotes tumorigenesis through regulating AKT/mTOR pathway. *Oncotarget* 2017; **8**: 27929-27942 [PMID: 28427191 DOI: 10.18632/oncotarget.15846]

19 **Thorenoor N**, Faltejskova-Vychytilova P, Hombach S, Mlcochova J, Kretz M, Svoboda M, Slaby O. Long non-coding RNA ZFAS1 interacts with CDK1 and is involved in p53-dependent cell cycle control and apoptosis in colorectal cancer. *Oncotarget* 2016; **7**: 622-637 [PMID: 26506418 DOI: 10.18632/oncotarget.5807]

20 **Yang P**, Yang Y, An W, Xu J, Zhang G, Jie J, Zhang Q. The long noncoding RNA-ROR promotes the resistance of radiotherapy for human colorectal cancer cells by targeting the p53/miR-145 pathway. *J Gastroenterol Hepatol* 2017; **32**: 837-845 [PMID: 27696511 DOI: 10.1111/jgh.13606]

21 **Qi P**, Zhou XY, Du X. Circulating long non-coding RNAs in cancer: current status and future perspectives. *Mol Cancer* 2016; **15**: 39 [PMID: 27189224 DOI: 10.1186/s12943-016-0524-4]

22 **Yang Y**, Junjie P, Sanjun C, Ma Y. Long non-coding RNAs in Colorectal Cancer: Progression and Future Directions. *J Cancer* 2017; **8**: 3212-3225 [PMID: 29158793 DOI: 10.7150/jca.19794]

23 **Alaiyan B**, Ilyayev N, Stojadinovic A, Izadjoo M, Roistacher M, Pavlov V, Tzivin V, Halle D, Pan H, Trink B, Gure AO, Nissan A. Differential expression of colon cancer associated transcript1 (CCAT1) along the colonic adenoma-carcinoma sequence. *BMC Cancer* 2013; **13**: 196 [PMID: 23594791 DOI: 10.1186/1471-2407-13-196]

24 **Graham LD**, Pedersen SK, Brown GS, Ho T, Kassir Z, Moynihan AT, Vizgoft EK, Dunne R, Pimlott L, Young GP, Lapointe LC, Molloy PL. Colorectal Neoplasia Differentially Expressed (CRNDE), a Novel Gene with Elevated Expression in Colorectal Adenomas and Adenocarcinomas. *Genes Cancer* 2011; **2**: 829-840 [PMID: 22393467 DOI: 10.1177/1947601911431081]

25 **Pachnis V**, Belayew A, Tilghman SM. Locus unlinked to alpha-fetoprotein under the control of the murine raf and Rif genes. *Proc Natl Acad Sci USA* 1984; **81**: 5523-5527 [PMID: 6206499 DOI: 10.1073/pnas.81.17.5523]

26 **Borsani G**, Tonlorenzi R, Simmler MC, Dandolo L, Arnaud D, Capra V, Grompe M, Pizzuti A, Muzny D, Lawrence C, Willard HF, Avner P, Ballabio A. Characterization of a murine gene expressed from the inactive X chromosome. *Nature* 1991; **351**: 325-329 [PMID: 2034278 DOI: 10.1038/351325a0]

27 **Jarroux J**, Morillon A, Pinskaya M. History, Discovery, and Classification of lncRNAs. *Adv Exp Med Biol* 2017; **1008**: 1-46 [PMID: 28815535 DOI: 10.1007/978-981-10-5203-3_1]

28 **Kung JT**, Colognori D, Lee JT. Long noncoding RNAs: past, present, and future. *Genetics* 2013; **193**:

651-669 [PMID: 23463798 DOI: 10.1534/genetics.112.146704]
29 **Gloss BS**, Dinger ME. Realizing the significance of noncoding functionality in clinical genomics. *Exp Mol Med* 2018; **50**: 97 [PMID: 30089779 DOI: 10.1038/s12276-018-0087-0]
30 **Cheng L**, Wang P, Tian R, Wang S, Guo Q, Luo M, Zhou W, Liu G, Jiang H, Jiang Q. LncRNA2Target v2.0: a comprehensive database for target genes of lncRNAs in human and mouse. *Nucleic Acids Res* 2019; **47**: D140-D144 [PMID: 30380072 DOI: 10.1093/nar/gky1051]
31 **Lee RC**, Feinbaum RL, Ambros V. The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. *Cell* 1993; **75**: 843-854 [PMID: 8252621]
32 **Mercer TR**, Dinger ME, Mattick JS. Long non-coding RNAs: insights into functions. *Nat Rev Genet* 2009; **10**: 155-159 [PMID: 19188922 DOI: 10.1038/nrg2521]
33 **Ulitsky I**, Bartel DP. lincRNAs: genomics, evolution, and mechanisms. *Cell* 2013; **154**: 26-46 [PMID: 23827673 DOI: 10.1016/j.cell.2013.06.020]
34 **Pelechano V**, Steinmetz LM. Gene regulation by antisense transcription. *Nat Rev Genet* 2013; **14**: 880-893 [PMID: 24217315 DOI: 10.1038/nrg3594]
35 **Ma L**, Bajic VB, Zhang Z. On the classification of long non-coding RNAs. *RNA Biol* 2013; **10**: 925-933 [PMID: 23696037 DOI: 10.4161/rna.24604]
36 **Katayama S**, Tomaru Y, Kasukawa T, Waki K, Nakanishi M, Nakamura M, Nishida H, Yap CC, Suzuki M, Kawai J, Suzuki H, Carninci P, Hayashizaki Y, Wells C, Frith M, Ravasi T, Pang KC, Hallinan J, Mattick J, Hume DA, Lipovich L, Batalov S, Engström PG, Mizuno Y, Faghihi MA, Sandelin A, Chalk AM, Mottagui-Tabar S, Liang Z, Lenhard B, Wahlestedt C; RIKEN Genome Exploration Research Group; Genome Science Group (Genome Network Project Core Group); FANTOM Consortium. Antisense transcription in the mammalian transcriptome. *Science* 2005; **309**: 1564-1566 [PMID: 16141073 DOI: 10.1126/science.1112009]
37 **He Y**, Vogelstein B, Velculescu VE, Papadopoulos N, Kinzler KW. The antisense transcriptomes of human cells. *Science* 2008; **322**: 1855-1857 [PMID: 19056939 DOI: 10.1126/science.1163853]
38 **Lee JT**, Davidow LS, Warshawsky D. Tsix, a gene antisense to Xist at the X-inactivation centre. *Nat Genet* 1999; **21**: 400-404 [PMID: 10192391 DOI: 10.1038/7734]
39 **Balakirev ES**, Ayala FJ. Pseudogenes: are they "junk" or functional DNA? *Annu Rev Genet* 2003; **37**: 123-151 [PMID: 14616058 DOI: 10.1146/annurev.genet.37.040103.103949]
40 **St Laurent G**, Wahlestedt C, Kapranov P. The Landscape of long noncoding RNA classification. *Trends Genet* 2015; **31**: 239-251 [PMID: 25869999 DOI: 10.1016/j.tig.2015.03.007]
41 **Geisler S**, Coller J. RNA in unexpected places: long non-coding RNA functions in diverse cellular contexts. *Nat Rev Mol Cell Biol* 2013; **14**: 699-712 [PMID: 24105322 DOI: 10.1038/nrm3679]
42 **Gudenas BL**, Wang L. Prediction of LncRNA Subcellular Localization with Deep Learning from Sequence Features. *Sci Rep* 2018; **8**: 16385 [PMID: 30401954 DOI: 10.1038/s41598-018-34708-w]
43 **Cech TR**, Steitz JA. The noncoding RNA revolution-trashing old rules to forge new ones. *Cell* 2014; **157**: 77-94 [PMID: 24679528 DOI: 10.1016/j.cell.2014.03.008]
44 **Long Y**, Wang X, Youmans DT, Cech TR. How do lncRNAs regulate transcription? *Sci Adv* 2017; **3**: eaao2110 [PMID: 28959731 DOI: 10.1126/sciadv.aao2110]
45 **Kornienko AE**, Guenzl PM, Barlow DP, Pauler FM. Gene regulation by the act of long non-coding RNA transcription. *BMC Biol* 2013; **11**: 59 [PMID: 23721193 DOI: 10.1186/1741-7007-11-59]
46 **Yao RW**, Wang Y, Chen LL. Cellular functions of long noncoding RNAs. *Nat Cell Biol* 2019; **21**: 542-551 [PMID: 31048766 DOI: 10.1038/s41556-019-0311-8]
47 **Yang S**, Sun Z, Zhou Q, Wang W, Wang G, Song J, Li Z, Zhang Z, Chang Y, Xia K, Liu J, Yuan W. MicroRNAs, long noncoding RNAs, and circular RNAs: potential tumor biomarkers and targets for colorectal cancer. *Cancer Manag Res* 2018; **10**: 2249-2257 [PMID: 30100756 DOI: 10.2147/CMAR.S166308]
48 **Romero-Barrios N**, Legascue MF, Benhamed M, Ariel F, Crespi M. Splicing regulation by long noncoding RNAs. *Nucleic Acids Res* 2018; **46**: 2169-2184 [PMID: 29425321 DOI: 10.1093/nar/gky095]
49 **Willingham AT**, Orth AP, Batalov S, Peters EC, Wen BG, Aza-Blanc P, Hogenesch JB, Schultz PG. A strategy for probing the function of noncoding RNAs finds a repressor of NFAT. *Science* 2005; **309**: 1570-1573 [PMID: 16141075 DOI: 10.1126/science.1115901]
50 **Staněk D**, Fox AH. Nuclear bodies: news insights into structure and function. *Curr Opin Cell Biol* 2017; **46**: 94-101 [PMID: 28577509 DOI: 10.1016/j.ceb.2017.05.001]
51 **Chujo T**, Yamazaki T, Hirose T. Architectural RNAs (arcRNAs): A class of long noncoding RNAs that function as the scaffold of nuclear bodies. *Biochim Biophys Acta* 2016; **1859**: 139-146 [PMID: 26021608 DOI: 10.1016/j.bbagrm.2015.05.007]
52 **Clemson CM**, Hutchinson JN, Sara SA, Ensminger AW, Fox AH, Chess A, Lawrence JB. An architectural role for a nuclear noncoding RNA: NEAT1 RNA is essential for the structure of paraspeckles. *Mol Cell* 2009; **33**: 717-726 [PMID: 19217333 DOI: 10.1016/j.molcel.2009.01.026]
53 **Tsai MC**, Manor O, Wan Y, Mosammaparast N, Wang JK, Lan F, Shi Y, Segal E, Chang HY. Long noncoding RNA as modular scaffold of histone modification complexes. *Science* 2010; **329**: 689-693 [PMID: 20616235 DOI: 10.1126/science.1192002]
54 **Merry CR**, Forrest ME, Sabers JN, Beard L, Gao XH, Hatzoglou M, Jackson MW, Wang Z, Markowitz SD, Khalil AM. DNMT1-associated long non-coding RNAs regulate global gene expression and DNA methylation in colon cancer. *Hum Mol Genet* 2015; **24**: 6240-6253 [PMID: 26307088 DOI: 10.1093/hmg/ddv343]
55 **Zhao Y**, Sun H, Wang H. Long noncoding RNAs in DNA methylation: new players stepping into the old game. *Cell Biosci* 2016; **6**: 45 [PMID: 27408682 DOI: 10.1186/s13578-016-0109-3]
56 **Clark MB**, Johnston RL, Inostroza-Ponta M, Fox AH, Fortini E, Moscato P, Dinger ME, Mattick JS. Genome-wide analysis of long noncoding RNA stability. *Genome Res* 2012; **22**: 885-898 [PMID: 22406755 DOI: 10.1101/gr.131037.111]
57 **Sharova LV**, Sharov AA, Nedorezov T, Piao Y, Shaik N, Ko MS. Database for mRNA half-life of 19 977 genes obtained by DNA microarray analysis of pluripotent and differentiating mouse embryonic stem cells. *DNA Res* 2009; **16**: 45-58 [PMID: 19001483 DOI: 10.1093/dnares/dsn030]
58 **Tani H**, Mizutani R, Salam KA, Tano K, Ijiri K, Wakamatsu A, Isogai T, Suzuki Y, Akimitsu N. Genome-wide determination of RNA stability reveals hundreds of short-lived noncoding transcripts in mammals. *Genome Res* 2012; **22**: 947-956 [PMID: 22369889 DOI: 10.1101/gr.130559.111]
59 **Zampetaki A**, Albrecht A, Steinhofel K. Long Non-coding RNA Structure and Function: Is There a Link? *Front Physiol* 2018; **9**: 1201 [PMID: 30197605 DOI: 10.3389/fphys.2018.01201]
60 **Dinger ME**, Amaral PP, Mercer TR, Mattick JS. Pervasive transcription of the eukaryotic genome:

functional indices and conceptual implications. *Brief Funct Genomic Proteomic* 2009; **8**: 407-423 [PMID: 19770204 DOI: 10.1093/bfgp/elp038]
61 **Mao YS**, Sunwoo H, Zhang B, Spector DL. Direct visualization of the co-transcriptional assembly of a nuclear body by noncoding RNAs. *Nat Cell Biol* 2011; **13**: 95-101 [PMID: 21170033 DOI: 10.1038/ncb2140]
62 **Charles Richard JL**, Eichhorn PJA. Platforms for Investigating LncRNA Functions. *SLAS Technol* 2018; **23**: 493-506 [PMID: 29945466 DOI: 10.1177/2472630318780639]
63 **Velculescu VE**, Zhang L, Vogelstein B, Kinzler KW. Serial analysis of gene expression. *Science* 1995; **270**: 484-487 [PMID: 7570003]
64 **Kashi K**, Henderson L, Bonetti A, Carninci P. Discovery and functional analysis of lncRNAs: Methodologies to investigate an uncharacterized transcriptome. *Biochim Biophys Acta* 2016; **1859**: 3-15 [PMID: 26477492 DOI: 10.1016/j.bbagrm.2015.10.010]
65 **Wang Z**, Gerstein M, Snyder M. RNA-Seq: a revolutionary tool for transcriptomics. *Nat Rev Genet* 2009; **10**: 57-63 [PMID: 19015660 DOI: 10.1038/nrg2484]
66 **Shiraki T**, Kondo S, Katayama S, Waki K, Kasukawa T, Kawaji H, Kodzius R, Watahiki A, Nakamura M, Arakawa T, Fukuda S, Sasaki D, Podhajska A, Harbers M, Kawai J, Carninci P, Hayashizaki Y. Cap analysis gene expression for high-throughput analysis of transcriptional starting point and identification of promoter usage. *Proc Natl Acad Sci USA* 2003; **100**: 15776-15781 [PMID: 14663149 DOI: 10.1073/pnas.2136655100]
67 **Ma J**, Chen Q, Ma D. Biological Functions and Research Methods of Long Noncoding RNAs. *Shengzhi Yu Biyun* 2017; **1**: 23-29
68 **Ferrè F**, Colantoni A, Helmer-Citterich M. Revealing protein-lncRNA interaction. *Brief Bioinform* 2016; **17**: 106-116 [PMID: 26041786 DOI: 10.1093/bib/bbv031]
69 **Uren PJ**, Bahrami-Samani E, Burns SC, Qiao M, Karginov FV, Hodges E, Hannon GJ, Sanford JR, Penalva LO, Smith AD. Site identification in high-throughput RNA-protein interaction data. *Bioinformatics* 2012; **28**: 3013-3020 [PMID: 23024010 DOI: 10.1093/bioinformatics/bts569]
70 **Konig J**, Zarnack K, Rot G, Curk T, Kayikci M, Zupan B, Turner DJ, Luscombe NM, Ule J. iCLIP--transcriptome-wide mapping of protein-RNA interactions with individual nucleotide resolution. *J Vis Exp* 2011 [PMID: 21559008 DOI: 10.3791/2638]
71 **Chu C**, Quinn J, Chang HY. Chromatin isolation by RNA purification (ChIRP). *J Vis Exp* 2012; **pii**: 3912 [PMID: 22472705 DOI: 10.3791/3912]
72 **Simon MD**, Wang CI, Kharchenko PV, West JA, Chapman BA, Alekseyenko AA, Borowsky ML, Kuroda MI, Kingston RE. The genomic binding sites of a noncoding RNA. *Proc Natl Acad Sci USA* 2011; **108**: 20497-20502 [PMID: 22143764 DOI: 10.1073/pnas.1113536108]
73 **Engreitz J**, Lander ES, Guttman M. RNA antisense purification (RAP) for mapping RNA interactions with chromatin. *Methods Mol Biol* 2015; **1262**: 183-197 [PMID: 25555582 DOI: 10.1007/978-1-4939-2253-6_11]
74 **Engreitz JM**, Sirokman K, McDonel P, Shishkin AA, Surka C, Russell P, Grossman SR, Chow AY, Guttman M, Lander ES. RNA-RNA interactions enable specific targeting of noncoding RNAs to nascent Pre-mRNAs and chromatin sites. *Cell* 2014; **159**: 188-199 [PMID: 25259926 DOI: 10.1016/j.cell.2014.08.018]
75 **Kudla G**, Granneman S, Hahn D, Beggs JD, Tollervey D. Cross-linking, ligation, and sequencing of hybrids reveals RNA-RNA interactions in yeast. *Proc Natl Acad Sci USA* 2011; **108**: 10010-10015 [PMID: 21610164 DOI: 10.1073/pnas.1017386108]
76 **Wan Y**, Kertesz M, Spitale RC, Segal E, Chang HY. Understanding the transcriptome through RNA structure. *Nat Rev Genet* 2011; **12**: 641-655 [PMID: 21850044 DOI: 10.1038/nrg3049]
77 **Shah S**, Takei Y, Zhou W, Lubeck E, Yun J, Eng CL, Koulena N, Cronin C, Karp C, Liaw EJ, Amin M, Cai L. Dynamics and Spatial Genomics of the Nascent Transcriptome by Intron seqFISH. *Cell* 2018; **174**: 363-376.e16 [PMID: 29887381 DOI: 10.1016/j.cell.2018.05.035]
78 **Chen KH**, Boettiger AN, Moffitt JR, Wang S, Zhuang X. RNA imaging. Spatially resolved, highly multiplexed RNA profiling in single cells. *Science* 2015; **348**: aaa6090 [PMID: 25858977 DOI: 10.1126/science.aaa6090]
79 **Cabili MN**, Dunagin MC, McClanahan PD, Biaesch A, Padovan-Merhar O, Regev A, Rinn JL, Raj A. Localization and abundance analysis of human lncRNAs at single-cell and single-molecule resolution. *Genome Biol* 2015; **16**: 20 [PMID: 25630241 DOI: 10.1186/s13059-015-0586-4]
80 **Gustafsson MG**. Nonlinear structured-illumination microscopy: wide-field fluorescence imaging with theoretically unlimited resolution. *Proc Natl Acad Sci U S A* 2005; **102**: 13081-13086 [PMID: 16141335 DOI: 10.1073/pnas.0406877102]
81 **Rust MJ**, Bates M, Zhuang X. Sub-diffraction-limit imaging by stochastic optical reconstruction microscopy (STORM). *Nat Methods* 2006; **3**: 793-795 [PMID: 16896339 DOI: 10.1038/nmeth929]
82 **Dias N**, Stein CA. Potential roles of antisense oligonucleotides in cancer therapy. The example of Bcl-2 antisense oligonucleotides. *Eur J Pharm Biopharm* 2002; **54**: 263-269 [PMID: 12445555]
83 **Jinek M**, Chylinski K, Fonfara I, Hauer M, Doudna JA, Charpentier E. A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science* 2012; **337**: 816-821 [PMID: 22745249 DOI: 10.1126/science.1225829]
84 **Liu SJ**, Horlbeck MA, Cho SW, Birk HS, Malatesta M, He D, Attenello FJ, Villalta JE, Cho MY, Chen Y, Mandegar MA, Olvera MP, Gilbert LA, Conklin BR, Chang HY, Weissman JS, Lim DA. CRISPRi-based genome-scale identification of functional long noncoding RNA loci in human cells. *Science* 2017; 355 [PMID: 27980086 DOI: 10.1126/science.aah7111]
85 **Bester AC**, Lee JD, Chavez A, Lee YR, Nachmani D, Vora S, Victor J, Sauvageau M, Monteleone E, Rinn JL, Provero P, Church GM, Clohessy JG, Pandolfi PP. An Integrated Genome-wide CRISPRa Approach to Functionalize lncRNAs in Drug Resistance. *Cell* 2018; **173**: 649-664.e20 [PMID: 29677511 DOI: 10.1016/j.cell.2018.03.052]
86 **Gilbert LA**, Horlbeck MA, Adamson B, Villalta JE, Chen Y, Whitehead EH, Guimaraes C, Panning B, Ploegh HL, Bassik MC, Qi LS, Kampmann M, Weissman JS. Genome-Scale CRISPR-Mediated Control of Gene Repression and Activation. *Cell* 2014; **159**: 647-661 [PMID: 25307932 DOI: 10.1016/j.cell.2014.09.029]
87 **Gupta RA**, Shah N, Wang KC, Kim J, Horlings HM, Wong DJ, Tsai MC, Hung T, Argani P, Rinn JL, Wang Y, Brzoska P, Kong B, Li R, West RB, van de Vijver MJ, Sukumar S, Chang HY. Long non-coding RNA HOTAIR reprograms chromatin state to promote cancer metastasis. *Nature* 2010; **464**: 1071-1076 [PMID: 20393566 DOI: 10.1038/nature08975]

88 **Wu ZH**, Wang XL, Tang HM, Jiang T, Chen J, Lu S, Qiu GQ, Peng ZH, Yan DW. Long non-coding RNA HOTAIR is a powerful predictor of metastasis and poor prognosis and is associated with epithelial-mesenchymal transition in colon cancer. *Oncol Rep* 2014; **32**: 395-402 [PMID: 24840737 DOI: 10.3892/or.2014.3186]

89 **Kogo R**, Shimamura T, Mimori K, Kawahara K, Imoto S, Sudo T, Tanaka F, Shibata K, Suzuki A, Komune S, Miyano S, Mori M. Long noncoding RNA HOTAIR regulates polycomb-dependent chromatin modification and is associated with poor prognosis in colorectal cancers. *Cancer Res* 2011; **71**: 6320-6326 [PMID: 21862635 DOI: 10.1158/0008-5472.CAN-11-1021]

90 **Svoboda M**, Slyskova J, Schneiderova M, Makovicky P, Bielik L, Levy M, Lipska L, Hemmelova B, Kala Z, Protivankova M, Vycital O, Liska V, Schwarzova L, Vodickova L, Vodicka P. HOTAIR long non-coding RNA is a negative prognostic factor not only in primary tumors, but also in the blood of colorectal cancer patients. *Carcinogenesis* 2014; **35**: 1510-1515 [PMID: 24583926 DOI: 10.1093/carcin/bgu055]

91 **Xu C**, Yang M, Tian J, Wang X, Li Z. MALAT-1: a long non-coding RNA and its important 3' end functional motif in colorectal cancer metastasis. *Int J Oncol* 2011; **39**: 169-175 [PMID: 21503572 DOI: 10.3892/ijo.2011.1007]

92 **Zheng HT**, Shi DB, Wang YW, Li XX, Xu Y, Tripathi P, Gu WL, Cai GX, Cai SJ. High expression of lncRNA MALAT1 suggests a biomarker of poor prognosis in colorectal cancer. *Int J Clin Exp Pathol* 2014; **7**: 3174-3181 [PMID: 25031737]

93 **Ji Q**, Zhang L, Liu X, Zhou L, Wang W, Han Z, Sui H, Tang Y, Wang Y, Liu N, Ren J, Hou F, Li Q. Long non-coding RNA MALAT1 promotes tumour growth and metastasis in colorectal cancer through binding to SFPQ and releasing oncogene PTBP2 from SFPQ/PTBP2 complex. *Br J Cancer* 2014; **111**: 736-748 [PMID: 25025966 DOI: 10.1038/bjc.2014.383]

94 **Tsang WP**, Ng EK, Ng SS, Jin H, Yu J, Sung JJ, Kwok TT. Oncofetal H19-derived miR-675 regulates tumor suppressor RB in human colorectal cancer. *Carcinogenesis* 2010; **31**: 350-358 [PMID: 19926638 DOI: 10.1093/carcin/bgp181]

95 **Yang W**, Ning N, Jin X. The lncRNA H19 Promotes Cell Proliferation by Competitively Binding to miR-200a and Derepressing *β*-Catenin Expression in Colorectal Cancer. *Biomed Res Int* 2017; **2017**: 2767484 [PMID: 28164117 DOI: 10.1155/2017/2767484]

96 **Nissan A**, Stojadinovic A, Mitrani-Rosenbaum S, Halle D, Grinbaum R, Roistacher M, Bochem A, Dayanc BE, Ritter G, Gomceli I, Bostanci EB, Akoglu M, Chen YT, Old LJ, Gure AO. Colon cancer associated transcript-1: a novel RNA expressed in malignant and pre-malignant human tissues. *Int J Cancer* 2012; **130**: 1598-1606 [PMID: 21547902 DOI: 10.1002/ijc.26170]

97 **He X**, Tan X, Wang X, Jin H, Liu L, Ma L, Yu H, Fan Z. C-Myc-activated long noncoding RNA CCAT1 promotes colon cancer cell proliferation and invasion. *Tumour Biol* 2014; **35**: 12181-12188 [PMID: 25185650 DOI: 10.1007/s13277-014-2526-4]

98 **Xie X**, Tang B, Xiao YF, Xie R, Li BS, Dong H, Zhou JY, Yang SM. Long non-coding RNAs in colorectal cancer. *Oncotarget* 2016; **7**: 5226-5239 [PMID: 26637808 DOI: 10.18632/oncotarget.6446]

99 **Liu T**, Zhang X, Yang YM, Du LT, Wang CX. Increased expression of the long noncoding RNA CRNDE-h indicates a poor prognosis in colorectal cancer, and is positively correlated with IRX5 mRNA expression. *Onco Targets Ther* 2016; **9**: 1437-1448 [PMID: 27042112 DOI: 10.2147/ott.s98268]

100 **Yamada A**, Yu P, Lin W, Okugawa Y, Boland CR, Goel A. A RNA-Sequencing approach for the identification of novel long non-coding RNA biomarkers in colorectal cancer. *Sci Rep* 2018; **8**: 575 [PMID: 29330370 DOI: 10.1038/s41598-017-18407-6]

101 **Barbagallo C**, Brex D, Caponnetto A, Cirnigliaro M, Scalia M, Magnano A, Caltabiano R, Barbagallo D, Biondi A, Cappellani A, Basile F, Di Pietro C, Purrello M, Ragusa M. LncRNA UCA1, Upregulated in CRC Biopsies and Downregulated in Serum Exosomes, Controls mRNA Expression by RNA-RNA Interactions. *Mol Ther Nucleic Acids* 2018; **12**: 229-241 [PMID: 30195762 DOI: 10.1016/j.omtn.2018.05.009]

102 **Kalmar A**, Nagy ZB, Galamb O, Csabai I, Bodor A, Wichmann B, Valcz G, Bartak BK, Tulassay Z, Igaz P, Molnar B. Genome-wide expression profiling in colorectal cancer focusing on lncRNAs in the adenoma-carcinoma transition. *BMC Cancer* 2019; In press

103 **Liu T**, Zhang X, Gao S, Jing F, Yang Y, Du L, Zheng G, Li P, Li C, Wang C. Exosomal long noncoding RNA CRNDE-h as a novel serum-based biomarker for diagnosis and prognosis of colorectal cancer. *Oncotarget* 2016; **7**: 85551-85563 [PMID: 27888803 DOI: 10.18632/oncotarget.13465]

104 **Ding J**, Li J, Wang H, Tian Y, Xie M, He X, Ji H, Ma Z, Hui B, Wang K, Ji G. Long noncoding RNA CRNDE promotes colorectal cancer cell proliferation via epigenetically silencing DUSP5/CDKN1A expression. *Cell Death Dis* 2017; **8**: e2997 [PMID: 28796262 DOI: 10.1038/cddis.2017.328]

105 **Jiang H**, Wang Y, Ai M, Wang H, Duan Z, Wang H, Zhao L, Yu J, Ding Y, Wang S. Long noncoding RNA CRNDE stabilized by hnRNPUL2 accelerates cell proliferation and migration in colorectal carcinoma via activating Ras/MAPK signaling pathways. *Cell Death Dis* 2017; **8**: e2862 [PMID: 28594403 DOI: 10.1038/cddis.2017.258]

106 **Yu B**, Ye X, Du Q, Zhu B, Zhai Q, Li XX. The Long Non-Coding RNA CRNDE Promotes Colorectal Carcinoma Progression by Competitively Binding miR-217 with TCF7L2 and Enhancing the Wnt/β-Catenin Signaling Pathway. *Cell Physiol Biochem* 2017; **41**: 2489-2502 [PMID: 28472810 DOI: 10.1159/000475941]

107 **Han P**, Li JW, Zhang BM, Lv JC, Li YM, Gu XY, Yu ZW, Jia YH, Bai XF, Li L, Liu YL, Cui BB. The lncRNA CRNDE promotes colorectal cancer cell proliferation and chemoresistance via miR-181a-5p-mediated regulation of Wnt/β-catenin signaling. *Mol Cancer* 2017; **16**: 9 [PMID: 28086904 DOI: 10.1186/s12943-017-0583-1]

108 **Gao H**, Song X, Kang T, Yan B, Feng L, Gao L, Ai L, Liu X, Yu J, Li H. Long noncoding RNA *CRNDE* functions as a competing endogenous RNA to promote metastasis and oxaliplatin resistance by sponging miR-136 in colorectal cancer. *Onco Targets Ther* 2017; **10**: 205-216 [PMID: 28115855 DOI: 10.2147/OTT.S116178]

109 **Tao K**, Yang J, Hu Y, Sun Y, Tan Z, Duan J, Zhang F, Yan H, Deng A. Clinical significance of urothelial carcinoma associated 1 in colon cancer. *Int J Clin Exp Med* 2015; **8**: 21854-21860 [PMID: 26885155]

110 **Han Y**, Yang YN, Yuan HH, Zhang TT, Sui H, Wei XL, Liu L, Huang P, Zhang WJ, Bai YX. UCA1, a long non-coding RNA up-regulated in colorectal cancer influences cell proliferation, apoptosis and cell cycle distribution. *Pathology* 2014; **46**: 396-401 [PMID: 24977734 DOI: 10.1097/PAT.0000000000000125]

111 **Lao Y**, Li Q, Li N, Liu H, Liu K, Jiang G, Wei N, Wang C, Wang Y, Wu J. Long noncoding RNA ENST00000455974 plays an oncogenic role through up-regulating JAG2 in human DNA mismatch repair-

proficient colon cancer. *Biochem Biophys Res Commun* 2019; **508**: 339-347 [PMID: 30473216 DOI: 10.1016/j.bbrc.2018.11.088]
112 **Sole C**, Arnaiz E, Manterola L, Otaegui D, Lawrie CH. The circulating transcriptome as a source of cancer liquid biopsy biomarkers. *Semin Cancer Biol* 2019 [PMID: 30684535 DOI: 10.1016/j.semcancer.2019.01.003]
113 **Li Q**, Shao Y, Zhang X, Zheng T, Miao M, Qin L, Wang B, Ye G, Xiao B, Guo J. Plasma long noncoding RNA protected by exosomes as a potential stable biomarker for gastric cancer. *Tumour Biol* 2015; **36**: 2007-2012 [PMID: 25391424 DOI: 10.1007/s13277-014-2807-y]
114 **Viereck J**, Thum T. Circulating Noncoding RNAs as Biomarkers of Cardiovascular Disease and Injury. *Circ Res* 2017; **120**: 381-399 [PMID: 28104771 DOI: 10.1161/CIRCRESAHA.116.308434]
115 **Chen D**, Sun Q, Cheng X, Zhang L, Song W, Zhou D, Lin J, Wang W. Genome-wide analysis of long noncoding RNA (lncRNA) expression in colorectal cancer tissues from patients with liver metastasis. *Cancer Med* 2016; **5**: 1629-1639 [PMID: 27165481 DOI: 10.1002/cam4.738]
116 **Zhao W**, Song M, Zhang J, Kuerban M, Wang H. Combined identification of long non-coding RNA CCAT1 and HOTAIR in serum as an effective screening for colorectal carcinoma. *Int J Clin Exp Pathol* 2015; **8**: 14131-14140 [PMID: 26823726]
117 **Wan L**, Kong J, Tang J, Wu Y, Xu E, Lai M, Zhang H. HOTAIRM1 as a potential biomarker for diagnosis of colorectal cancer functions the role in the tumour suppressor. *J Cell Mol Med* 2016; **20**: 2036-2044 [PMID: 27307307 DOI: 10.1111/jcmm.12892]
118 **Li Y**, Huang S, Li Y, Zhang W, He K, Zhao M, Lin H, Li D, Zhang H, Zheng Z, Huang C. Decreased expression of LncRNA SLC25A25-AS1 promotes proliferation, chemoresistance, and EMT in colorectal cancer cells. *Tumour Biol* 2016; **37**: 14205-14215 [PMID: 27553025 DOI: 10.1007/s13277-016-5254-0]
119 **Ye C**, Shen Z, Wang B, Li Y, Li T, Yang Y, Jiang K, Ye Y, Wang S. A novel long non-coding RNA lnc-GNAT1-1 is low expressed in colorectal cancer and acts as a tumor suppressor through regulating RKIP-NF-κB-Snail circuit. *J Exp Clin Cancer Res* 2016; **35**: 187 [PMID: 27912775 DOI: 10.1186/s13046-016-0467-z]
120 **Li Y**, Li Y, Huang S, He K, Zhao M, Lin H, Li D, Qian J, Zhou C, Chen Y, Huang C. Long non-coding RNA growth arrest specific transcript 5 acts as a tumour suppressor in colorectal cancer by inhibiting interleukin-10 and vascular endothelial growth factor expression. *Oncotarget* 2017; **8**: 13690-13702 [PMID: 28099146 DOI: 10.18632/oncotarget.14625]
121 **Cao D**, Ding Q, Yu W, Gao M, Wang Y. Long noncoding RNA SPRY4-IT1 promotes malignant development of colorectal cancer by targeting epithelial-mesenchymal transition. *Onco Targets Ther* 2016; **9**: 5417-5425 [PMID: 27621655 DOI: 10.2147/OTT.S111794]
122 **Yue B**, Qiu S, Zhao S, Liu C, Zhang D, Yu F, Peng Z, Yan D. LncRNA-ATB mediated E-cadherin repression promotes the progression of colon cancer and predicts poor prognosis. *J Gastroenterol Hepatol* 2016; **31**: 595-603 [PMID: 26487301 DOI: 10.1111/jgh.13206]
123 **Yue B**, Sun B, Liu C, Zhao S, Zhang D, Yu F, Yan D. Long non-coding RNA Fer-1-like protein 4 suppresses oncogenesis and exhibits prognostic value by associating with miR-106a-5p in colon cancer. *Cancer Sci* 2015; **106**: 1323-1332 [PMID: 26224446 DOI: 10.1111/cas.12759]
124 **Wang C**, Yu J, Han Y, Li L, Li J, Li T, Qi P. Long non-coding RNAs LOC285194, RP11-462C24.1 and Nbla12061 in serum provide a new approach for distinguishing patients with colorectal cancer from healthy controls. *Oncotarget* 2016; **7**: 70769-70778 [PMID: 27683121 DOI: 10.18632/oncotarget.12220]
125 **Wang R**, Du L, Yang X, Jiang X, Duan W, Yan S, Xie Y, Zhu Y, Wang Q, Wang L, Yang Y, Wang C. Identification of long noncoding RNAs as potential novel diagnosis and prognosis biomarkers in colorectal cancer. *J Cancer Res Clin Oncol* 2016; **142**: 2291-2301 [PMID: 27591862 DOI: 10.1007/s00432-016-2238-9]
126 **Shi J**, Li X, Zhang F, Zhang C, Guan Q, Cao X, Zhu W, Zhang X, Cheng Y, Ou K, Chen Q, Hu S. Circulating lncRNAs associated with occurrence of colorectal cancer progression. *Am J Cancer Res* 2015; **5**: 2258-2265 [PMID: 26328256]
127 **Xu Y**, Xu Q, Yang L, Ye X, Liu F, Wu F, Ni S, Tan C, Cai G, Meng X, Cai S, Du X. Identification and validation of a blood-based 18-gene expression signature in colorectal cancer. *Clin Cancer Res* 2013; **19**: 3039-3049 [PMID: 23536436 DOI: 10.1158/1078-0432.CCR-12-3851]
128 **Wu Y**, Yang L, Zhao J, Li C, Nie J, Liu F, Zhuo C, Zheng Y, Li B, Wang Z, Xu Y. Nuclear-enriched abundant transcript 1 as a diagnostic and prognostic biomarker in colorectal cancer. *Mol Cancer* 2015; **14**: 191 [PMID: 26552600 DOI: 10.1186/s12943-015-0455-5]
129 **Dai M**, Chen X, Mo S, Li J, Huang Z, Huang S, Xu J, He B, Zou Y, Chen J, Dai S. Meta-signature LncRNAs serve as novel biomarkers for colorectal cancer: integrated bioinformatics analysis, experimental validation and diagnostic evaluation. *Sci Rep* 2017; **7**: 46572 [PMID: 28406230 DOI: 10.1038/srep46572]
130 **Liu H**, Ye D, Chen A, Tan D, Zhang W, Jiang W, Wang M, Zhang X. A pilot study of new promising non-coding RNA diagnostic biomarkers for early-stage colorectal cancers. *Clin Chem Lab Med* 2019; **57**: 1073-1083 [PMID: 30978169 DOI: 10.1515/cclm-2019-0052]
131 **Pedersen SK**, Mitchell SM, Graham LD, McEvoy A, Thomas ML, Baker RT, Ross JP, Xu ZZ, Ho T, LaPointe LC, Young GP, Molloy PL. CAHM, a long non-coding RNA gene hypermethylated in colorectal neoplasia. *Epigenetics* 2014; **9**: 1071-1082 [PMID: 24799664 DOI: 10.4161/epi.29046]
132 **Gong W**, Tian M, Qiu H, Yang Z. Elevated serum level of lncRNA-HIF1A-AS1 as a novel diagnostic predictor for worse prognosis in colorectal carcinoma. *Cancer Biomark* 2017; **20**: 417-424 [PMID: 28946548 DOI: 10.3233/CBM-170179]
133 **Wang L**, Du L, Duan W, Yan S, Xie Y, Wang C. Overexpression of long noncoding RNA NORAD in colorectal cancer associates with tumor progression. *Onco Targets Ther* 2018; **11**: 6757-6766 [PMID: 30349308 DOI: 10.2147/OTT.S176354]
134 **Shaker OG**, Senousy MA, Elbaz EM. Association of rs6983267 at 8q24, HULC rs7763881 polymorphisms and serum lncRNAs CCAT2 and HULC with colorectal cancer in Egyptian patients. *Sci Rep* 2017; **7**: 16246 [PMID: 29176650 DOI: 10.1038/s41598-017-16500-4]
135 **Kalluri R**. The biology and function of exosomes in cancer. *J Clin Invest* 2016; **126**: 1208-1215 [PMID: 27035812 DOI: 10.1172/JCI81135]
136 **Shao Y**, Shen Y, Chen T, Xu F, Chen X, Zheng S. The functions and clinical applications of tumor-derived exosomes. *Oncotarget* 2016; **7**: 60736-60751 [PMID: 27517627 DOI: 10.18632/oncotarget.11177]
137 **Azmi AS**, Bao B, Sarkar FH. Exosomes in cancer development, metastasis, and drug resistance: a comprehensive review. *Cancer Metastasis Rev* 2013; **32**: 623-642 [PMID: 23709120 DOI: 10.1007/s10555-013-9441-9]

138 **Gao T**, Liu X, He B, Nie Z, Zhu C, Zhang P, Wang S. Exosomal lncRNA 91H is associated with poor development in colorectal cancer by modifying HNRNPK expression. *Cancer Cell Int* 2018; **18**: 11 [PMID: 29410604 DOI: 10.1186/s12935-018-0506-2]

139 **Hu D**, Zhan Y, Zhu K, Bai M, Han J, Si Y, Zhang H, Kong D. Plasma Exosomal Long Non-Coding RNAs Serve as Biomarkers for Early Detection of Colorectal Cancer. *Cell Physiol Biochem* 2018; **51**: 2704-2715 [PMID: 30562751 DOI: 10.1159/000495961]

140 **Dong L**, Lin W, Qi P, Xu MD, Wu X, Ni S, Huang D, Weng WW, Tan C, Sheng W, Zhou X, Du X. Circulating Long RNAs in Serum Extracellular Vesicles: Their Characterization and Potential Application as Biomarkers for Diagnosis of Colorectal Cancer. *Cancer Epidemiol Biomarkers Prev* 2016; **25**: 1158-1166 [PMID: 27197301 DOI: 10.1158/1055-9965.EPI-16-0006]

141 **Liu L**, Meng T, Yang XH, Sayim P, Lei C, Jin B, Ge L, Wang HJ. Prognostic and predictive value of long non-coding RNA GAS5 and mircoRNA-221 in colorectal cancer and their effects on colorectal cancer cell proliferation, migration and invasion. *Cancer Biomark* 2018; **22**: 283-299 [PMID: 29630521 DOI: 10.3233/CBM-171011]

142 **Molnár B**, Galamb O, Kalmár A, Barták BK, Nagy ZB, Tóth K, Tulassay Z, Igaz P, Dank M. Circulating cell-free nucleic acids as biomarkers in colorectal cancer screening and diagnosis - an update. *Expert Rev Mol Diagn* 2019; **19**: 477-498 [PMID: 31046485 DOI: 10.1080/14737159.2019.1613891]

143 **Ohtsuka M**, Ling H, Ivan C, Pichler M, Matsushita D, Goblirsch M, Stiegelbauer V, Shigeyasu K, Zhang X, Chen M, Vidhu F, Bartholomeusz GA, Toiyama Y, Kusunoki M, Doki Y, Mori M, Song S, Gunther JR, Krishnan S, Slaby O, Goel A, Ajani JA, Radovich M, Calin GA. H19 Noncoding RNA, an Independent Prognostic Factor, Regulates Essential Rb-E2F and CDK8-β-Catenin Signaling in Colorectal Cancer. *EBioMedicine* 2016; **13**: 113-124 [PMID: 27789274 DOI: 10.1016/j.ebiom.2016.10.026]

144 **Han D**, Gao X, Wang M, Qiao Y, Xu Y, Yang J, Dong N, He J, Sun Q, Lv G, Xu C, Tao J, Ma N. Long noncoding RNA H19 indicates a poor prognosis of colorectal cancer and promotes tumor growth by recruiting and binding to eIF4A3. *Oncotarget* 2016; **7**: 22159-22173 [PMID: 26989025 DOI: 10.18632/oncotarget.8063]

145 **Li Q**, Dai Y, Wang F, Hou S. Differentially expressed long non-coding RNAs and the prognostic potential in colorectal cancer. *Neoplasma* 2016; **63**: 977-983 [PMID: 27596298 DOI: 10.4149/neo_2016_617]

146 **Yu B**, Du Q, Li H, Liu HY, Ye X, Zhu B, Zhai Q, Li XX. Diagnostic potential of serum exosomal colorectal neoplasia differentially expressed long non-coding RNA (CRNDE-p) and microRNA-217 expression in colorectal carcinoma. *Oncotarget* 2017; **8**: 83745-83753 [PMID: 29137379 DOI: 10.18632/oncotarget.19407]

147 **Li P**, Zhang X, Wang L, Du L, Yang Y, Liu T, Li C, Wang C. lncRNA HOTAIR Contributes to 5FU Resistance through Suppressing miR-218 and Activating NF-κB/TS Signaling in Colorectal Cancer. *Mol Ther Nucleic Acids* 2017; **8**: 356-369 [PMID: 28918035 DOI: 10.1016/j.omtn.2017.07.007]

148 **Xiao Z**, Qu Z, Chen Z, Fang Z, Zhou K, Huang Z, Guo X, Zhang Y. LncRNA HOTAIR is a Prognostic Biomarker for the Proliferation and Chemoresistance of Colorectal Cancer via MiR-203a-3p-Mediated Wnt/ß-Catenin Signaling Pathway. *Cell Physiol Biochem* 2018; **46**: 1275-1285 [PMID: 29680837 DOI: 10.1159/000489110]

149 **Li P**, Zhang X, Wang H, Wang L, Liu T, Du L, Yang Y, Wang C. MALAT1 Is Associated with Poor Response to Oxaliplatin-Based Chemotherapy in Colorectal Cancer Patients and Promotes Chemoresistance through EZH2. *Mol Cancer Ther* 2017; **16**: 739-751 [PMID: 28069878 DOI: 10.1158/1535-7163.MCT-16-0591]

150 **Ozawa T**, Matsuyama T, Toiyama Y, Takahashi N, Ishikawa T, Uetake H, Yamada Y, Kusunoki M, Calin G, Goel A. CCAT1 and CCAT2 long noncoding RNAs, located within the 8q.24.21 'gene desert', serve as important prognostic biomarkers in colorectal cancer. *Ann Oncol* 2017; **28**: 1882-1888 [PMID: 28838211 DOI: 10.1093/annonc/mdx248]

151 **Su J**, Zhang E, Han L, Yin D, Liu Z, He X, Zhang Y, Lin F, Lin Q, Mao P, Mao W, Shen D. Long noncoding RNA BLACAT1 indicates a poor prognosis of colorectal cancer and affects cell proliferation by epigenetically silencing of p15. *Cell Death Dis* 2017; **8**: e2665 [PMID: 28277544 DOI: 10.1038/cddis.2017.83]

152 **Deng Q**, He B, Gao T, Pan Y, Sun H, Xu Y, Li R, Ying H, Wang F, Liu X, Chen J, Wang S. Up-regulation of 91H promotes tumor metastasis and predicts poor prognosis for patients with colorectal cancer. *PLoS One* 2014; **9**: e103022 [PMID: 25058480 DOI: 10.1371/journal.pone.0103022]

153 **Han X**, Wang L, Ning Y, Li S, Wang Z. Long non-coding RNA AFAP1-AS1 facilitates tumor growth and promotes metastasis in colorectal cancer. *Biol Res* 2016; **49**: 36 [PMID: 27578191 DOI: 10.1186/s40659-016-0094-3]

154 **Wang F**, Ni H, Sun F, Li M, Chen L. Overexpression of lncRNA AFAP1-AS1 correlates with poor prognosis and promotes tumorigenesis in colorectal cancer. *Biomed Pharmacother* 2016; **81**: 152-159 [PMID: 27261589 DOI: 10.1016/j.biopha.2016.04.009]

155 **Niu H**, Hu Z, Liu H, Hu G, Yang B, Wu S, Li F. Long non-coding RNA AK027294 involves in the process of proliferation, migration, and apoptosis of colorectal cancer cells. *Tumour Biol* 2016; **37**: 10097-10105 [PMID: 26820130 DOI: 10.1007/s13277-015-4350-x]

156 **Hu Y**, Chen HY, Yu CY, Xu J, Wang JL, Qian J, Zhang X, Fang JY. A long non-coding RNA signature to improve prognosis prediction of colorectal cancer. *Oncotarget* 2014; **5**: 2230-2242 [PMID: 24809982 DOI: 10.18632/oncotarget.1895]

157 **Han J**, Rong LF, Shi CB, Dong XG, Wang J, Wang BL, Wen H, He ZY. Screening of lymph nodes metastasis associated lncRNAs in colorectal cancer patients. *World J Gastroenterol* 2014; **20**: 8139-8150 [PMID: 25009386 DOI: 10.3748/wjg.v20.i25.8139]

158 **Sun Y**, Zheng ZP, Li H, Zhang HQ, Ma FQ. ANRIL is associated with the survival rate of patients with colorectal cancer, and affects cell migration and invasion in vitro. *Mol Med Rep* 2016; **14**: 1714-1720 [PMID: 27314206 DOI: 10.3892/mmr.2016.5409]

159 **Naemura M**, Tsunoda T, Inoue Y, Okamoto H, Shirasawa S, Kotake Y. ANRIL regulates the proliferation of human colorectal cancer cells in both two- and three-dimensional culture. *Mol Cell Biochem* 2016; **412**: 141-146 [PMID: 26708220 DOI: 10.1007/s11010-015-2618-5]

160 **Iguchi T**, Uchi R, Nambara S, Saito T, Komatsu H, Hirata H, Ueda M, Sakimura S, Takano Y, Kurashige J, Shinden Y, Eguchi H, Sugimachi K, Maehara Y, Mimori K. A long noncoding RNA, lncRNA-ATB, is involved in the progression and prognosis of colorectal cancer. *Anticancer Res* 2015; **35**: 1385-1388 [PMID: 25750289]

161 **Li W**, Kang Y. A new Lnc in metastasis: long noncoding RNA mediates the prometastatic functions of TGF-β. *Cancer Cell* 2014; **25**: 557-559 [PMID: 24823634 DOI: 10.1016/j.ccr.2014.04.014]

162 **Davison EJ**, Tarpey PS, Fiegler H, Tomlinson IP, Carter NP. Deletion at chromosome band 20p12.1 in colorectal cancer revealed by high resolution array comparative genomic hybridization. *Genes Chromosomes Cancer* 2005; **44**: 384-391 [PMID: 16110499 DOI: 10.1002/gcc.20252]

163 **Shi Y**, Liu Y, Wang J, Jie D, Yun T, Li W, Yan L, Wang K, Feng J. Downregulated Long Noncoding RNA BANCR Promotes the Proliferation of Colorectal Cancer Cells via Downregualtion of p21 Expression. *PLoS One* 2015; **10**: e0122679 [PMID: 25928067 DOI: 10.1371/journal.pone.0122679]

164 **Guo Q**, Zhao Y, Chen J, Hu J, Wang S, Zhang D, Sun Y. BRAF-activated long non-coding RNA contributes to colorectal cancer migration by inducing epithelial-mesenchymal transition. *Oncol Lett* 2014; **8**: 869-875 [PMID: 25013510 DOI: 10.3892/ol.2014.2154]

165 **Gao X**, Wen J, Gao P, Zhang G, Zhang G. Overexpression of the long non-coding RNA, linc-UBC1, is associated with poor prognosis and facilitates cell proliferation, migration, and invasion in colorectal cancer. *Onco Targets Ther* 2017; **10**: 1017-1026 [PMID: 28260919 DOI: 10.2147/OTT.S129343]

166 **Zhang Z**, Zhou C, Chang Y, Zhang Z, Hu Y, Zhang F, Lu Y, Zheng L, Zhang W, Li X, Li X. Long non-coding RNA CASC11 interacts with hnRNP-K and activates the WNT/β-catenin pathway to promote growth and metastasis in colorectal cancer. *Cancer Lett* 2016; **376**: 62-73 [PMID: 27012187 DOI: 10.1016/j.canlet.2016.03.022]

167 **Huang G**, Wu X, Li S, Xu X, Zhu H, Chen X. The long noncoding RNA CASC2 functions as a competing endogenous RNA by sponging miR-18a in colorectal cancer. *Sci Rep* 2016; **6**: 26524 [PMID: 27198161 DOI: 10.1038/srep26524]

168 **Xin Y**, Li Z, Shen J, Chan MT, Wu WK. CCAT1: a pivotal oncogenic long non-coding RNA in human cancers. *Cell Prolif* 2016; **49**: 255-260 [PMID: 27134049 DOI: 10.1111/cpr.12252]

169 **McCleland ML**, Mesh K, Lorenzana E, Chopra VS, Segal E, Watanabe C, Haley B, Mayba O, Yaylaoglu M, Gnad F, Firestein R. CCAT1 is an enhancer-templated RNA that predicts BET sensitivity in colorectal cancer. *J Clin Invest* 2016; **126**: 639-652 [PMID: 26752646 DOI: 10.1172/JCI83265]

170 **Ye Z**, Zhou M, Tian B, Wu B, Li J. Expression of lncRNA-CCAT1, E-cadherin and N-cadherin in colorectal cancer and its clinical significance. *Int J Clin Exp Med* 2015; **8**: 3707-3715 [PMID: 26064266]

171 **Xiang JF**, Yin QF, Chen T, Zhang Y, Zhang XO, Wu Z, Zhang S, Wang HB, Ge J, Lu X, Yang L, Chen LL. Human colorectal cancer-specific CCAT1-L lncRNA regulates long-range chromatin interactions at the MYC locus. *Cell Res* 2014; **24**: 513-531 [PMID: 24662484 DOI: 10.1038/cr.2014.35]

172 **Ling H**, Spizzo R, Atlasi Y, Nicoloso M, Shimizu M, Redis RS, Nishida N, Gafà R, Song J, Guo Z, Ivan C, Barbarotto E, De Vries I, Zhang X, Ferracin M, Churchman M, van Galen JF, Beverloo BH, Shariati M, Haderk F, Estecio MR, Garcia-Manero G, Patijn GA, Gotley DC, Bhardwaj V, Shureiqi I, Sen S, Multani AS, Welsh J, Yamamoto K, Taniguchi I, Song MA, Gallinger S, Casey G, Thibodeau SN, Le Marchand L, Tiirikainen M, Mani SA, Zhang W, Davuluri RV, Mimori K, Mori M, Sieuwerts AM, Martens JW, Tomlinson I, Negrini M, Berindan-Neagoe I, Foekens JA, Hamilton SR, Lanza G, Kopetz S, Fodde R, Calin GA. CCAT2, a novel noncoding RNA mapping to 8q24, underlies metastatic progression and chromosomal instability in colon cancer. *Genome Res* 2013; **23**: 1446-1461 [PMID: 23796952 DOI: 10.1101/gr.152942.112]

173 **Kasagi Y**, Oki E, Ando K, Ito S, Iguchi T, Sugiyama M, Nakashima Y, Ohgaki K, Saeki H, Mimori K, Maehara Y. The Expression of CCAT2, a Novel Long Noncoding RNA Transcript, and rs6983267 Single-Nucleotide Polymorphism Genotypes in Colorectal Cancers. *Oncology* 2017; **92**: 48-54 [PMID: 27875818 DOI: 10.1159/000452143]

174 **Ye LC**, Ren L, Qiu JJ, Zhu DX, Chen T, Chang WJ, Lv SX, Xu J. Aberrant expression of long noncoding RNAs in colorectal cancer with liver metastasis. *Tumour Biol* 2015; **36**: 8747-8754 [PMID: 26050227 DOI: 10.1007/s13277-015-3627-4]

175 **Ye LC**, Chen T, Zhu DX, Lv SX, Qiu JJ, Xu J, Yuan FL, Wei Y. Downregulated long non-coding RNA CLMAT3 promotes the proliferation of colorectal cancer cells by targeting regulators of the cell cycle pathway. *Oncotarget* 2016; **7**: 58931-58938 [PMID: 27391344 DOI: 10.18632/oncotarget.10431]

176 **Yuan Z**, Yu X, Ni B, Chen D, Yang Z, Huang J, Wang J, Chen D, Wang L. Overexpression of long non-coding RNA-CTD903 inhibits colorectal cancer invasion and migration by repressing Wnt/β-catenin signaling and predicts favorable prognosis. *Int J Oncol* 2016; **48**: 2675-2685 [PMID: 27035092 DOI: 10.3892/ijo.2016.3447]

177 **Chen X**, Zhu H, Wu X, Xie X, Huang G, Xu X, Li S, Xing C. Downregulated pseudogene CTNNAP1 promote tumor growth in human cancer by downregulating its cognate gene CTNNA1 expression. *Oncotarget* 2016; **7**: 55518-55528 [PMID: 27487124 DOI: 10.18632/oncotarget.10833]

178 **Liu Y**, Zhang M, Liang L, Li J, Chen YX. Over-expression of lncRNA DANCR is associated with advanced tumor progression and poor prognosis in patients with colorectal cancer. *Int J Clin Exp Pathol* 2015; **8**: 11480-11484 [PMID: 26617879]

179 **Sun L**, Xue H, Jiang C, Zhou H, Gu L, Liu Y, Xu C, Xu Q. LncRNA DQ786243 contributes to proliferation and metastasis of colorectal cancer both in vitro and in vivo. *Biosci Rep* 2016; 36 [PMID: 26934980 DOI: 10.1042/bsr20160048]

180 **Yochum GS**, Cleland R, McWeeney S, Goodman RH. An antisense transcript induced by Wnt/beta-catenin signaling decreases E2F4. *J Biol Chem* 2007; **282**: 871-878 [PMID: 17121828 DOI: 10.1074/jbc.M609391200]

181 **Yang P**, Xu ZP, Chen T, He ZY. Long noncoding RNA expression profile analysis of colorectal cancer and metastatic lymph node based on microarray data. *Onco Targets Ther* 2016; **9**: 2465-2478 [PMID: 27217770 DOI: 10.2147/OTT.S102348]

182 **Chen N**, Guo D, Xu Q, Yang M, Wang D, Peng M, Ding Y, Wang S, Zhou J. Long non-coding RNA FEZF1-AS1 facilitates cell proliferation and migration in colorectal carcinoma. *Oncotarget* 2016; **7**: 11271-11283 [PMID: 26848625 DOI: 10.18632/oncotarget.7168]

183 **Guo XB**, Hua Z, Li C, Peng LP, Wang JS, Wang B, Zhi QM. Biological significance of long non-coding RNA FTX expression in human colorectal cancer. *Int J Clin Exp Med* 2015; **8**: 15591-15600 [PMID: 26629053]

184 **Yang P**, Chen T, Xu Z, Zhu H, Wang J, He Z. Long noncoding RNA GAPLINC promotes invasion in colorectal cancer by targeting SNAI2 through binding with PSF and NONO. *Oncotarget* 2016; **7**: 42183-42194 [PMID: 27259250 DOI: 10.18632/oncotarget.9741]

185 **Yin D**, He X, Zhang E, Kong R, De W, Zhang Z. Long noncoding RNA GAS5 affects cell proliferation and predicts a poor prognosis in patients with colorectal cancer. *Med Oncol* 2014; **31**: 253 [PMID: 25326054 DOI: 10.1007/s12032-014-0253-8]

186 **Ma C**, Shi X, Zhu Q, Li Q, Liu Y, Yao Y, Song Y. The growth arrest-specific transcript 5 (GAS5): a pivotal tumor suppressor long noncoding RNA in human cancers. *Tumour Biol* 2016; **37**: 1437-1444

[PMID: 26634743 DOI: 10.1007/s13277-015-4521-9]
187 **Krell J**, Frampton AE, Mirnezami R, Harding V, De Giorgio A, Roca Alonso L, Cohen P, Ottaviani S, Colombo T, Jacob J, Pellegrino L, Buchanan G, Stebbing J, Castellano L. Growth arrest-specific transcript 5 associated snoRNA levels are related to p53 expression and DNA damage in colorectal cancer. *PLoS One* 2014; **9**: e98561 [PMID: 24926850 DOI: 10.1371/journal.pone.0098561]
188 **Zheng Y**, Song D, Xiao K, Yang C, Ding Y, Deng W, Tong S. LncRNA GAS5 contributes to lymphatic metastasis in colorectal cancer. *Oncotarget* 2016; **7**: 83727-83734 [PMID: 27863421 DOI: 10.18632/oncotarget.13384]
189 **Zhou J**, Li X, Wu M, Lin C, Guo Y, Tian B. Knockdown of Long Noncoding RNA GHET1 Inhibits Cell Proliferation and Invasion of Colorectal Cancer. *Oncol Res* 2016; **23**: 303-309 [PMID: 27131316]
190 **Liang WC**, Fu WM, Wong CW, Wang Y, Wang WM, Hu GX, Zhang L, Xiao LJ, Wan DC, Zhang JF, Waye MM. The lncRNA H19 promotes epithelial to mesenchymal transition by functioning as miRNA sponges in colorectal cancer. *Oncotarget* 2015; **6**: 22513-22525 [PMID: 26068968 DOI: 10.18632/oncotarget.4154]
191 **Wu KF**, Liang WC, Feng L, Pang JX, Waye MM, Zhang JF, Fu WM. H19 mediates methotrexate resistance in colorectal cancer through activating Wnt/β-catenin pathway. *Exp Cell Res* 2017; **350**: 312-317 [PMID: 27919747 DOI: 10.1016/j.yexcr.2016.12.003]
192 **Yao J**, Li J, Geng P, Li Y, Chen H, Zhu Y. Knockdown of a HIF-2α promoter upstream long noncoding RNA impairs colorectal cancer stem cell properties in vitro through HIF-2α downregulation. *Onco Targets Ther* 2015; **8**: 3467-3474 [PMID: 26648739 DOI: 10.2147/OTT.S81393]
193 **Dou J**, Ni Y, He X, Wu D, Li M, Wu S, Zhang R, Guo M, Zhao F. Decreasing lncRNA HOTAIR expression inhibits human colorectal cancer stem cells. *Am J Transl Res* 2016; **8**: 98-108 [PMID: 27069543]
194 **Luo ZF**, Zhao D, Li XQ, Cui YX, Ma N, Lu CX, Liu MY, Zhou Y. Clinical significance of HOTAIR expression in colon cancer. *World J Gastroenterol* 2016; **22**: 5254-5259 [PMID: 27298568 DOI: 10.3748/wjg.v22.i22.5254]
195 **Ren YK**, Xiao Y, Wan XB, Zhao YZ, Li J, Li Y, Han GS, Chen XB, Zou QY, Wang GC, Lu CM, Xu YC, Wang YC. Association of long non-coding RNA HOTTIP with progression and prognosis in colorectal cancer. *Int J Clin Exp Pathol* 2015; **8**: 11458-11463 [PMID: 26617875]
196 **Lian Y**, Ding J, Zhang Z, Shi Y, Zhu Y, Li J, Peng P, Wang J, Fan Y, De W, Wang K. The long noncoding RNA HOXA transcript at the distal tip promotes colorectal cancer growth partially via silencing of p21 expression. *Tumour Biol* 2016; **37**: 7431-7440 [PMID: 26678886 DOI: 10.1007/s13277-015-4617-2]
197 **Yang XJ**, Huang CQ, Peng CW, Hou JX, Liu JY. Long noncoding RNA HULC promotes colorectal carcinoma progression through epigenetically repressing NKD2 expression. *Gene* 2016; **592**: 172-178 [PMID: 27496341 DOI: 10.1016/j.gene.2016.08.002]
198 **Tanaka K**, Shiota G, Meguro M, Mitsuya K, Oshimura M, Kawasaki H. Loss of imprinting of long QT intronic transcript 1 in colorectal cancer. *Oncology* 2001; **60**: 268-273 [PMID: 11340379 DOI: 10.1159/000055328]
199 **Nakano S**, Murakami K, Meguro M, Soejima H, Higashimoto K, Urano T, Kugoh H, Mukai T, Ikeguchi M, Oshimura M. Expression profile of LIT1/KCNQ1OT1 and epigenetic status at the KvDMR1 in colorectal cancers. *Cancer Sci* 2006; **97**: 1147-1154 [PMID: 16965397 DOI: 10.1111/j.1349-7006.2006.00305.x]
200 **Kong J**, Sun W, Li C, Wan L, Wang S, Wu Y, Xu E, Zhang H, Lai M. Long non-coding RNA LINC01133 inhibits epithelial-mesenchymal transition and metastasis in colorectal cancer by interacting with SRSF6. *Cancer Lett* 2016; **380**: 476-484 [PMID: 27443606 DOI: 10.1016/j.canlet.2016.07.015]
201 **Qiu JJ**, Yan JB. Long non-coding RNA LINC01296 is a potential prognostic biomarker in patients with colorectal cancer. *Tumour Biol* 2015; **36**: 7175-7183 [PMID: 25894381 DOI: 10.1007/s13277-015-3448-5]
202 **Zhai H**, Fesler A, Schee K, Fodstad O, Flatmark K, Ju J. Clinical significance of long intergenic noncoding RNA-p21 in colorectal cancer. *Clin Colorectal Cancer* 2013; **12**: 261-266 [PMID: 24012455 DOI: 10.1016/j.clcc.2013.06.003]
203 **Wang J**, Lei ZJ, Guo Y, Wang T, Qin ZY, Xiao HL, Fan LL, Chen DF, Bian XW, Liu J, Wang B. miRNA-regulated delivery of lincRNA-p21 suppresses β-catenin signaling and tumorigenicity of colorectal cancer stem cells. *Oncotarget* 2015; **6**: 37852-37870 [PMID: 26497997 DOI: 10.18632/oncotarget.5635]
204 **Wang G**, Li Z, Zhao Q, Zhu Y, Zhao C, Li X, Ma Z, Li X, Zhang Y. LincRNA-p21 enhances the sensitivity of radiotherapy for human colorectal cancer by targeting the Wnt/β-catenin signaling pathway. *Oncol Rep* 2014; **31**: 1839-1845 [PMID: 24573322 DOI: 10.3892/or.2014.3047]
205 **Wang L**, Bu P, Ai Y, Srinivasan T, Chen HJ, Xiang K, Lipkin SM, Shen X. A long non-coding RNA targets microRNA miR-34a to regulate colon cancer stem cell asymmetric division. *Elife* 2016; 5 [PMID: 27077950 DOI: 10.7554/eLife.14620]
206 **Yang F**, Huo XS, Yuan SX, Zhang L, Zhou WP, Wang F, Sun SH. Repression of the long noncoding RNA-LET by histone deacetylase 3 contributes to hypoxia-mediated metastasis. *Mol Cell* 2013; **49**: 1083-1096 [PMID: 23395002 DOI: 10.1016/j.molcel.2013.01.010]
207 **Kazemzadeh M**, Safaralizadeh R, Feizi MA, Ravanbakhsh R, Somi MH, Hashemzadeh S. LOC100287225, novel long intergenic non-coding RNA, misregulates in colorectal cancer. *Cancer Biomark* 2016; **16**: 499-505 [PMID: 27062707 DOI: 10.3233/CBM-160589]
208 **Kazemzadeh M**, Safaralizadeh R, Feizi MA, Somi MH, Shokoohi B. Misregulation of the dependence receptor DCC and its upstream lincRNA, LOC100287225, in colorectal cancer. *Tumori* 2017; **103**: 40-43 [PMID: 26429648 DOI: 10.5301/tj.5000426]
209 **Qi P**, Xu MD, Ni SJ, Huang D, Wei P, Tan C, Zhou XY, Du X. Low expression of LOC285194 is associated with poor prognosis in colorectal cancer. *J Transl Med* 2013; **11**: 122 [PMID: 23680400 DOI: 10.1186/1479-5876-11-122]
210 **Xu J**, Zhang R, Zhao J. The Novel Long Noncoding RNA TUSC7 Inhibits Proliferation by Sponging MiR-211 in Colorectal Cancer. *Cell Physiol Biochem* 2017; **41**: 635-644 [PMID: 28214867 DOI: 10.1159/000457938]
211 **Ding J**, Lu B, Wang J, Wang J, Shi Y, Lian Y, Zhu Y, Wang J, Fan Y, Wang Z, De W, Wang K. Long non-coding RNA Loc554202 induces apoptosis in colorectal cancer cells via the caspase cleavage cascades. *J Exp Clin Cancer Res* 2015; **34**: 100 [PMID: 26362196 DOI: 10.1186/s13046-015-0217-7]
212 **Yang L**, Wei H, Xiao HJ. Long non-coding RNA Loc554202 expression as a prognostic factor in patients with colorectal cancer. *Eur Rev Med Pharmacol Sci* 2016; **20**: 4243-4247 [PMID: 27831651]

213 **Yang MH**, Hu ZY, Xu C, Xie LY, Wang XY, Chen SY, Li ZG. MALAT1 promotes colorectal cancer cell proliferation/migration/invasion via PRKA kinase anchor protein 9. *Biochim Biophys Acta* 2015; **1852**: 166-174 [PMID: 25446987 DOI: 10.1016/j.bbadis.2014.11.013]

214 **Wang Y**, Xue D, Li Y, Pan X, Zhang X, Kuang B, Zhou M, Li X, Xiong W, Li G, Zeng Z, Yang T. The Long Noncoding RNA MALAT-1 is A Novel Biomarker in Various Cancers: A Meta-analysis Based on the GEO Database and Literature. *J Cancer* 2016; **7**: 991-1001 [PMID: 27313790 DOI: 10.7150/jca.14663]

215 **Li L**, Shang J, Zhang Y, Liu S, Peng Y, Zhou Z, Pan H, Wang X, Chen L, Zhao Q. MEG3 is a prognostic factor for CRC and promotes chemosensitivity by enhancing oxaliplatin-induced cell apoptosis. *Oncol Rep* 2017; **38**: 1383-1392 [PMID: 28731151 DOI: 10.3892/or.2017.5828]

216 **Yin DD**, Liu ZJ, Zhang E, Kong R, Zhang ZH, Guo RH. Decreased expression of long noncoding RNA MEG3 affects cell proliferation and predicts a poor prognosis in patients with colorectal cancer. *Tumour Biol* 2015; **36**: 4851-4859 [PMID: 25636452 DOI: 10.1007/s13277-015-3139-2]

217 **Kong H**, Wu Y, Zhu M, Zhai C, Qian J, Gao X, Wang S, Hou Y, Lu S, Zhu H. Long non-coding RNAs: novel prognostic biomarkers for liver metastases in patients with early stage colorectal cancer. *Oncotarget* 2016; **7**: 50428-50436 [PMID: 27391432 DOI: 10.18632/oncotarget.10416]

218 **Cao X**, Zhuang S, Hu Y, Xi L, Deng L, Sheng H, Shen W. Associations between polymorphisms of long non-coding RNA MEG3 and risk of colorectal cancer in Chinese. *Oncotarget* 2016; **7**: 19054-19059 [PMID: 26934323 DOI: 10.18632/oncotarget.7764]

219 **Franklin JL**, Rankin CR, Levy S, Snoddy JR, Zhang B, Washington MK, Thomson JM, Whitehead RH, Coffey RJ. Malignant transformation of colonic epithelial cells by a colon-derived long noncoding RNA. *Biochem Biophys Res Commun* 2013; **440**: 99-104 [PMID: 24045012 DOI: 10.1016/j.bbrc.2013.09.040]

220 **Qi P**, Xu MD, Ni SJ, Shen XH, Wei P, Huang D, Tan C, Sheng WQ, Zhou XY, Du X. Down-regulation of ncRAN, a long non-coding RNA, contributes to colorectal cancer cell migration and invasion and predicts poor overall survival for colorectal cancer patients. *Mol Carcinog* 2015; **54**: 742-750 [PMID: 24519959 DOI: 10.1002/mc.22137]

221 **Smolle M**, Uranitsch S, Gerger A, Pichler M, Haybaeck J. Current status of long non-coding RNAs in human cancer with specific focus on colorectal cancer. *Int J Mol Sci* 2014; **15**: 13993-14013 [PMID: 25119862 DOI: 10.3390/ijms150813993]

222 **Yan B**, Gu W, Yang Z, Gu Z, Yue X, Gu Q, Liu L. Downregulation of a long noncoding RNA-ncRuPAR contributes to tumor inhibition in colorectal cancer. *Tumour Biol* 2014; **35**: 11329-11335 [PMID: 25119598 DOI: 10.1007/s13277-014-2465-0]

223 **Li Y**, Li Y, Chen W, He F, Tan Z, Zheng J, Wang W, Zhao Q, Li J. NEAT expression is associated with tumor recurrence and unfavorable prognosis in colorectal cancer. *Oncotarget* 2015; **6**: 27641-27650 [PMID: 26314847 DOI: 10.18632/oncotarget.4737]

224 **Lu M**, Liu Z, Li B, Wang G, Li D, Zhu Y. The high expression of long non-coding RNA PANDAR indicates a poor prognosis for colorectal cancer and promotes metastasis by EMT pathway. *J Cancer Res Clin Oncol* 2017; **143**: 71-81 [PMID: 27629879 DOI: 10.1007/s00432-016-2252-y]

225 **Ge X**, Chen Y, Liao X, Liu D, Li F, Ruan H, Jia W. Overexpression of long noncoding RNA PCAT-1 is a novel biomarker of poor prognosis in patients with colorectal cancer. *Med Oncol* 2013; **30**: 588 [PMID: 23640607 DOI: 10.1007/s12032-013-0588-6]

226 **Yang L**, Qiu M, Xu Y, Wang J, Zheng Y, Li M, Xu L, Yin R. Upregulation of long non-coding RNA PRNCR1 in colorectal cancer promotes cell proliferation and cell cycle progression. *Oncol Rep* 2016; **35**: 318-324 [PMID: 26530130 DOI: 10.3892/or.2015.4364]

227 **Li L**, Sun R, Liang Y, Pan X, Li Z, Bai P, Zeng X, Zhang D, Zhang L, Gao L. Association between polymorphisms in long non-coding RNA PRNCR1 in 8q24 and risk of colorectal cancer. *J Exp Clin Cancer Res* 2013; **32**: 104 [PMID: 24330491 DOI: 10.1186/1756-9966-32-104]

228 **Takahashi Y**, Sawada G, Kurashige J, Uchi R, Matsumura T, Ueo H, Takano Y, Eguchi H, Sudo T, Sugimachi K, Yamamoto H, Doki Y, Mori M, Mimori K. Amplification of PVT-1 is involved in poor prognosis via apoptosis inhibition in colorectal cancers. *Br J Cancer* 2014; **110**: 164-171 [PMID: 24196785 DOI: 10.1038/bjc.2013.698]

229 **Liu J**, Wang J, Song Y, Yang Y, Hu Y, Gao P, Sun J, Chen X, Xu Y, Wang Z. A potential biomarker for colorectal cancer: long non-coding RNA RP1-13P20.6. *Neoplasma* 2016; **63**: 984-990 [PMID: 27596299 DOI: 10.4149/neo_2016_618]

230 **Chen H**, Xu J, Hong J, Tang R, Zhang X, Fang JY. Long noncoding RNA profiles identify five distinct molecular subtypes of colorectal cancer with clinical relevance. *Mol Oncol* 2014; **8**: 1393-1403 [PMID: 24954858 DOI: 10.1016/j.molonc.2014.05.010]

231 **Shi D**, Zheng H, Zhuo C, Peng J, Li D, Xu Y, Li X, Cai G, Cai S. Low expression of novel lncRNA RP11-462C24.1 suggests a biomarker of poor prognosis in colorectal cancer. *Med Oncol* 2014; **31**: 31 [PMID: 24908062 DOI: 10.1007/s12032-014-0031-7]

232 **Lee H**, Kim C, Ku JL, Kim W, Yoon SK, Kuh HJ, Lee JH, Nam SW, Lee EK. A long non-coding RNA snaR contributes to 5-fluorouracil resistance in human colon cancer cells. *Mol Cells* 2014; **37**: 540-546 [PMID: 25078450 DOI: 10.14348/molcells.2014.0151]

233 **Li C**, Zhou L, He J, Fang XQ, Zhu SW, Xiong MM. Increased long noncoding RNA SNHG20 predicts poor prognosis in colorectal cancer. *BMC Cancer* 2016; **16**: 655 [PMID: 27543107 DOI: 10.1186/s12885-016-2719-x]

234 **Wang JZ**, Xu CL, Wu H, Shen SJ. LncRNA SNHG12 promotes cell growth and inhibits cell apoptosis in colorectal cancer cells. *Braz J Med Biol Res* 2017; **50**: e6079 [PMID: 28225893 DOI: 10.1590/1414-431X20176079]

235 **Liu S**, Xu B, Yan D. Enhanced expression of long non-coding RNA Sox2ot promoted cell proliferation and motility in colorectal cancer. *Minerva Med* 2016; **107**: 279-286 [PMID: 27353770]

236 **Shen F**, Cai WS, Feng Z, Chen JW, Feng JH, Liu QC, Fang YP, Li KP, Xiao HQ, Cao J, Xu B. Long non-coding RNA SPRY4-IT1 pormotes colorectal cancer metastasis by regulate epithelial-mesenchymal transition. *Oncotarget* 2017; **8**: 14479-14486 [PMID: 27391336 DOI: 10.18632/oncotarget.10407]

237 **Zhang ZY**, Lu YX, Zhang ZY, Chang YY, Zheng L, Yuan L, Zhang F, Hu YH, Zhang WJ, Li XN. Loss of TINCR expression promotes proliferation, metastasis through activating EpCAM cleavage in colorectal cancer. *Oncotarget* 2016; **7**: 22639-22649 [PMID: 27009809 DOI: 10.18632/oncotarget.8141]

238 **Sun J**, Ding C, Yang Z, Liu T, Zhang X, Zhao C, Wang J. The long non-coding RNA TUG1 indicates a poor prognosis for colorectal cancer and promotes metastasis by affecting epithelial-mesenchymal transition. *J Transl Med* 2016; **14**: 42 [PMID: 26856330 DOI: 10.1186/s12967-016-0786-z]

239 **Wang L**, Zhao Z, Feng W, Ye Z, Dai W, Zhang C, Peng J, Wu K. Long non-coding RNA TUG1 promotes

colorectal cancer metastasis via EMT pathway. *Oncotarget* 2016; **7**: 51713-51719 [PMID: 27421138 DOI: 10.18632/oncotarget.10563]

240 **Zhai HY**, Sui MH, Yu X, Qu Z, Hu JC, Sun HQ, Zheng HT, Zhou K, Jiang LX. Overexpression of Long Non-Coding RNA TUG1 Promotes Colon Cancer Progression. *Med Sci Monit* 2016; **22**: 3281-3287 [PMID: 27634385]

241 **Sana J**, Hankeova S, Svoboda M, Kiss I, Vyzula R, Slaby O. Expression levels of transcribed ultraconserved regions uc.73 and uc.388 are altered in colorectal cancer. *Oncology* 2012; **82**: 114-118 [PMID: 22328099 DOI: 10.1159/000336479]

242 **Calin GA**, Liu CG, Ferracin M, Hyslop T, Spizzo R, Sevignani C, Fabbri M, Cimmino A, Lee EJ, Wojcik SE, Shimizu M, Tili E, Rossi S, Taccioli C, Pichiorri F, Liu X, Zupo S, Herlea V, Gramantieri L, Lanza G, Alder H, Rassenti L, Volinia S, Schmittgen TD, Kipps TJ, Negrini M, Croce CM. Ultraconserved regions encoding ncRNAs are altered in human leukemias and carcinomas. *Cancer Cell* 2007; **12**: 215-229 [PMID: 17785203 DOI: 10.1016/j.ccr.2007.07.027]

243 **Wu X**, He X, Li S, Xu X, Chen X, Zhu H. Long Non-Coding RNA ucoo2kmd.1 Regulates CD44-Dependent Cell Growth by Competing for miR-211-3p in Colorectal Cancer. *PLoS One* 2016; **11**: e0151287 [PMID: 26974151 DOI: 10.1371/journal.pone.0151287]

244 **Bian Z**, Jin L, Zhang J, Yin Y, Quan C, Hu Y, Feng Y, Liu H, Fei B, Mao Y, Zhou L, Qi X, Huang S, Hua D, Xing C, Huang Z. LncRNA-UCA1 enhances cell proliferation and 5-fluorouracil resistance in colorectal cancer by inhibiting miR-204-5p. *Sci Rep* 2016; **6**: 23892 [PMID: 27046651 DOI: 10.1038/srep23892]

245 **Ni B**, Yu X, Guo X, Fan X, Yang Z, Wu P, Yuan Z, Deng Y, Wang J, Chen D, Wang L. Increased urothelial cancer associated 1 is associated with tumor proliferation and metastasis and predicts poor prognosis in colorectal cancer. *Int J Oncol* 2015; **47**: 1329-1338 [PMID: 26238511 DOI: 10.3892/ijo.2015.3109]

246 **Taniue K**, Kurimoto A, Sugimasa H, Nasu E, Takeda Y, Iwasaki K, Nagashima T, Okada-Hatakeyama M, Oyama M, Kozuka-Hata H, Hiyoshi M, Kitayama J, Negishi L, Kawasaki Y, Akiyama T. Long noncoding RNA UPAT promotes colon tumorigenesis by inhibiting degradation of UHRF1. *Proc Natl Acad Sci USA* 2016; **113**: 1273-1278 [PMID: 26768845 DOI: 10.1073/pnas.1500992113]

247 **Lassmann S**, Weis R, Makowiec F, Roth J, Danciu M, Hopt U, Werner M. Array CGH identifies distinct DNA copy number profiles of oncogenes and tumor suppressor genes in chromosomal- and microsatellite-unstable sporadic colorectal carcinomas. *J Mol Med (Berl)* 2007; **85**: 293-304 [PMID: 17143621 DOI: 10.1007/s00109-006-0126-5]

248 **Xiao Y**, Yurievich UA, Yosypovych SV. Long noncoding RNA XIST is a prognostic factor in colorectal cancer and inhibits 5-fluorouracil-induced cell cytotoxicity through promoting thymidylate synthase expression. *Oncotarget* 2017; **8**: 83171-83182 [PMID: 29137332 DOI: 10.18632/oncotarget.20487]

249 **Wang W**, Xing C. Upregulation of long noncoding RNA ZFAS1 predicts poor prognosis and prompts invasion and metastasis in colorectal cancer. *Pathol Res Pract* 2016; **212**: 690-695 [PMID: 27461828 DOI: 10.1016/j.prp.2016.05.003]

250 **Kumegawa K**, Maruyama R, Yamamoto E, Ashida M, Kitajima H, Tsuyada A, Niinuma T, Kai M, Yamano HO, Sugai T, Tokino T, Shinomura Y, Imai K, Suzuki H. A genomic screen for long noncoding RNA genes epigenetically silenced by aberrant DNA methylation in colorectal cancer. *Sci Rep* 2016; **6**: 26699 [PMID: 27215978 DOI: 10.1038/srep26699]

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REVIEW

Autoantibodies: Potential clinical applications in early detection of esophageal squamous cell carcinoma and esophagogastric junction adenocarcinoma

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Abstract

Esophageal squamous cell carcinoma (ESCC) and esophagogastric junction adenocarcinoma (EGJA) are the two main types of gastrointestinal cancers that pose a huge threat to human health. ESCC remains one of the most common malignant diseases around the world. In contrast to the decreasing prevalence of ESCC, the incidence of EGJA is rising rapidly. Early detection represents one of the most promising ways to improve the prognosis and reduce the mortality of these cancers. Current approaches for early diagnosis mainly depend on invasive and costly endoscopy. Non-invasive biomarkers are in great need to facilitate earlier detection for better clinical management of patients. Tumor-associated autoantibodies can be detected at an early stage before manifestations of clinical signs of tumorigenesis, making them promising biomarkers for early detection and monitoring of ESCC and EGJA. In this review, we summarize recent insights into the iden-tification and validation of tumor-associated autoantibodies for the early detection of ESCC and EGJA and discuss the challenges remaining for clinical validation.

Key words: Esophageal squamous cell carcinoma; Esophagogastric junction adenocarcinoma; Biomarker; Autoantibody; Diagnosis

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Core tip: The current protocol for early diagnosis of esophageal squamous cell carcinoma and esophagogastric junction adenocarcinoma is endoscopic imaging followed by biopsy confirmation. However, the invasive nature of the procedure and high cost of endoscopy limit it as a tool for screening the general population. This review highlights autoantibodies as non-invasive biomarkers in the early detection of esophageal squamous cell carcinoma and esophagogastric junction adenocarcinoma.

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INTRODUCTION

Esophageal cancer is the eighth leading malignant disease and the sixth most common cause of cancer-related death worldwide. It represents a serious health problem globally[1]. Esophageal cancer is mainly composed of two epidemiologically and histopathologically distinct sub-types designated as esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma. In China, esophageal cancer is the third leading cause of cancer death with an estimated 246000 new cases and 188000 deaths in 2015[2]. Although ESCC, which accounts for 70% of cases, remains the most prevalent form of esophageal cancer, the prevalence of ESCC has declined substantially in recent years. In contrast to the decreasing prevalence of ESCC, an alarming rise of the incidence in esophagogastric junction adenocarcinoma (EGJA) has been observed in both developed and developing countries with 260000 new cases diagnosed in 2012[2-4]. Interestingly, in China the incidence of EGJA appears to be high in areas where the prevalence of ESCC is also high[5]. This similar geographic distribution suggests similar environmental factors, similar dietary habits and even similar molecular alterations are involved in both ESCC and EGJA[6].

The prognosis of ESCC is poor with an overall 5-year incidence of survival ranging from 15% to 25%[7,8]. The high mortality in ESCC and EGJA mostly results from diagnosis at late stages due to the lack of specific symptoms of patients in early stage disease, but the prognosis is substantially better for patients diagnosed in the early stage (*e.g.*, 5-year survival of more than 85% for ESCC patients diagnosed in early stage and more than 90% for EGJA patients with node-negative T1 tumors)[9,10]. However, effective strategies are lacking for screening or detection of pre-cancerous lesions in early-stage ESCC and EGJA. Although endoscopy is used as a primary screening technique and can identify ESCC and EGJA at an early stage, its extensive utilization is limited by the invasive nature, serious side effects and dependence on the skill of the endoscopist. Moreover, some individuals are unwilling to undergo endoscopy, whereas a simple blood test might be more acceptable. Thus, identification and validation of novel non-invasive, blood-based biomarkers can fulfill a great need for early detection of ESCC and EGJA.

Tumor-associated (TA) autoantibodies are emerging as strong candidates for clinically useful cancer biomarkers because they are produced early in tumorigenesis and can be detectable up to five years before the clinical manifestations of cancer[11-13]. Moreover, autoantibodies are also reported as biomarkers used in cancer prognosis and therapeutic monitoring (Table 1)[11,14-21]. A large number of articles have evaluated the potential use of TA autoantibodies for early ESCC detection. Therefore, a systematic review is warranted to assess the current potential of TA autoantibodies for the early diagnosis of patients with ESCC. Considering the similarity of the etiology and epidemiology in EGJA and ESCC, we believe that it would be much desirable to provide together a review of TA autoantibodies in EGJA and ESCC at this current time. We focus on the key aspects of the study designs and participant characteristics, the sensitivity, specificity and area under the receiver operating characteristic curve of the TA autoantibody biomarkers to help identify the most promising candidates for future clinical screening tests.

Table 1 A brief summary of the biological significance of some common tumor-associated autoantibodies

Representative tumor-associated autoantigens	Authors, year	Tumor type	Biological significance
p53	Chapman *et al*[11], 2012	Lung	Early detection
	Takeda *et al*[14], 2001	Colorectal	Increased recurrence
	Anderson *et al*[15], 2010	Ovarian	Increased survival
NY-ESO-1	Shan *et al*[16], 2013	Lung	Early detection
	Fossså *et al*[17], 2004	Prostate	Decreased survival
	Elke *et al*[18], 1999	Melanoma	Therapeutic monitoring
MUC1	Pedersen *et al*[19], 2014	Ovarian	Early detection
	Kurtenkov *et al*[20], 2007	Gastric	Increased survival
Hu	Chapman *et al*[11], 2011	Lung	Early detection
	Graus *et al*[21], 1997	Lung	Therapeutic monitoring and increased survival

MUC1: Mucin-1.

PROPOSED ORIGINS OF AUTOANTIBODY PRODUCTION IN CANCER

As early as the 1960s, Robert W. Baldwin showed that the immune system could react to a developing tumor[22-24]. Most studies have mainly focused on evaluating TA autoantibodies as early cancer biomarkers since their discovery. On the other hand, investigation of the underlying causes of TA autoantibody production may contribute to a clearer understanding of mechanisms concerning the rendering of autologous proteins immunogenic and also reveal novel therapeutic targets for potential clinical use. It is commonly accepted that autologous cellular antigens expressed in tumors, also referred to as TA antigens (TAA), can be recognized early by the immune system and thus trigger a reaction known as cancer immunoediting, which consists of three phases: Elimination, equilibration and escape[25,26]. Immunosurveillance occurs during the elimination phase when the first few transformed cells are recognized by the immune system and targeted by natural killer cells that secrete certain cytokines to let other immune cells convene to the tumor[27]. The ensuing disruption of certain transformed cells and the uptake and disposal of the corresponding fragments by the recruited immune cells activate the appropriate immune response. A cascade of dynamic events further boosts the activation of innate immunity and facilitates the expansion and generation of T and B cells (the latter produces antibodies)[28]. Tumor cells that escape elimination and are permitted to grow will enter into the equilibrium phase during which tumor cell variants emerge with increasing ability to survive an immune attack. The equilibrium phase is the longest among these three phases and may persist for many years. Escape eventually occurs if the host immune defenses are breached and tumor cell variants grow and proliferate in an uncontrolled manner[29].

It is clear that the generation of many abnormal antigens during tumorigenesis can induce the host immune response to produce autoantibodies. However, how factors exactly facilitate an enhancement or disorder of immune surveillance in cancer resulting in the TA autoantibody production response is still unclear. The generation of TA autoantibodies is thought to occur in response to mutations[30,31], over-expression[32,33] or abnormal processing[34,35], which lead to the formation of altered or novel epitopes, aberrantly high expression levels resulting in loss of tolerance and abnormal post-translational modifications, such as acetylation, glycosylation and phosphorylation, all of which could create a neoepitope, enhance self-epitope presentation or expose antigens normally located in immune-privileged sites (*e.g.*, cancer-testis antigens). With these mechanisms, extracellular and intracellular host proteins could be recognized by B cells to produce TA autoantibodies. Recent research has estimated that most TA autoantigens are mutated or overexpressed proteins among which 42% are cytoplasmatic, 26.1% are expressed predominantly in the nucleus, 21.4% are membrane-bound and 10.3% are extracellular[36]. It is surprising that TA autoantibodies seem to be more specific to intracellular molecules rather than their more common cell surface targets. This may be explained by greater vascular permeability for cytoplasmic proteins and enhancement of autoantibody generation by the proinflammatory environment[37,38]. Although the exact role of TA auto-antibodies in cancer is largely undefined, that secreted TA autoantibodies reflect tumor burden makes them attractive and promising biomarkers.

DIAGNOSTIC PERFORMANCE OF SINGLE AUTOANTIBODIES IN ESCC

Although TA autoantibodies have been described in a wide variety of human malignancies in the past several decades and have shown early diagnostic relevance, most studies evaluating autoantibodies in patients with ESCC and normal controls have emerged within the last 20 years. At least 49 individual TA autoantibodies have been assessed with diagnostic parameters in ESCC. Table 2 represents a list of single TA autoantibodies reported in the literature that could serve as potential serum/plasma biomarkers for ESCC. The diagnostic value of the TA autoantibodies, whether for the same autoantibody or for different types of autoantibodies, shows large variation for ESCC in terms of sensitivity and specificity, which might be due to sample size, the ethnic group studied or the test method of evaluation.

In general, the majority of TA autoantibody biomarkers show relatively low sensitivity but high specificity. The sensitivities and specificities for ESCC range from 3.9% to 93.7% and from 78.7% to 100%, respectively (Table 2). Receiver operating characteristic curves as a summary measure will not set cutoff values artificially but rather considers sensitivity and specificity simultaneously. Nevertheless, only a few studies used receiver operating characteristic curve analysis and area under the curve values to evaluate the diagnostic performance of TA autoantibodies (Table 2). The graphical representation of the sensitivities and specificities for autoantibodies in ESCC evaluated in more than one study is shown in Figure 1. Of note, the diagnostic ability of the great majority of TA autoantibodies lack independent validation, and the numbers of cases in some of the studies are very small. It is also noteworthy that most of the single TA autoantibodies lack diagnostic assessment in patients with early stage ESCC, which is one of the most important elements for biomarker development and application in early cancer diagnosis.

The most comprehensively investigated TA autoantibodies in ESCC have been p53 autoantibodies followed by autoantibodies against P16 and c-Myc. Given the prominent feature of p53 in cancers it is not unexpected that this is the most widely studied autoantibody in ESCC. Autoantibodies against p53 in the diagnosis of ESCC have been evaluated in 17 studies (Table 2), and the sensitivities vary largely between reports (7%-60%) while less variance is observed in the specificity (range 89.5%-100%, Table 2). A meta-analysis by Zhang *et al*[39] showed the overall sensitivity and specificity of p53 autoantibody for esophageal cancer are 29.6% and 97.9%, respectively. Autoantibodies against P16 and c-Myc were each analyzed in five studies, and both exhibited high specificity but poor sensitivity (Table 2). Therefore, despite the high specificity, all studies show that use of a single autoantibody provides low sensitivity indicating limited clinical application. The sensitivity and specificity for Hsp70 autoantibodies reported by Fujita *et al*[40] can be up to 93.7% and 100%, respectively. However, the very small sample size of this study reduces the stability and power of the results. Overall, quite apparent is the fact that the diagnostic value of individual TA autoantibody biomarkers in ESCC is quite limited.

DIAGNOSTIC PERFORMANCE OF SINGLE AUTOANTIBODIES IN EGJA

Very few clinical or translational studies have treated EGJA as a separate entity, which have been generally divided between those targeting esophageal cancer and those targeting gastric cancer. Likewise, a similar phenomenon has been observed in the studies on autoantibodies for the diagnosis of EGJA. As can be seen from Table 3, a total of 13 autoantibodies were investigated in two studies[41,42], all of which were initially assessed in ESCC by Xu *et al*[43] and Zhou *et al*[44]. As anticipated, the presence of TA autoantibodies indicates early diagnostic potential for EGJA. The sensitivity of single TA autoantibody biomarkers for EGJA ranged from 11.0% to 54.3% with generally high specificity ranging from 86.3% to 97% (Table 3). From the list of autoantibodies shown in Table 3, there is no good way of forecasting which TA autoantibodies may work. Like ESCC, the most commonly tested TA autoantibody in EGJA is the p53 autoantibody, which has the highest area under the curve value (0.799) with moderate sensitivity and specificity in the diagnosis of early stage EGJA (Table 3). However, it remains fact that the capability of a single TA autoantibody biomarker to identify EGJA patients is limited. It also should be pointed out that research on autoantibodies is still in its infancy. Thus, more autoantibody biomarkers need to be identified and evaluated to enlarge the autoantibody pool for EGJA.

Table 2 Diagnostic performance of single tumor-associated autoantibody biomarkers in esophageal squamous cell carcinoma

Target antigen of auto-anti-bodies	Authors, year	ESCC cases, *n*	Stage, *n*					Con-trols, *n*	*P* value	Sensiti-vity, all stages/ early stage	Specifi-city, all stages/ early stage	AUC, all stages/ early stage	Method
			0/I	II	III	IV	Tx						
Survivin	Xiu *et al*[57], 2018	159	-	-	-	-	159	362	0.524	14.5%/-	90.0%/-	0.327/-	ELISA
	Qin *et al*[58], 2014	174	3/8	79	52	18	-	242	< 0.05	12.1%/-	99.6%/-	99.6/-	ELISA
	Zhou *et al*[44], 2014	88	-	-	-	-	88	200	0.06	9.0%/-	96.0%/-	-	ELISA
	Meglior-ino *et al*[59], 2005	77	-	-	-	-	77	82	< 0.05	10.4%/-	97.6%/-	-	ELISA
TOPO48	Zhang *et al*[60], 2018	112	29	28	28	27	-	112 healthy volunte-ers and 75 esopha-geal benign tumor patients	0.001	49.1%/ 61.4%	100%/ 100%	-/0.860	ELISA
L1CAM	Xu *et al*[61], 2017	191 (Cohort 1)	10	104	77	-	-	94 (Cohort 1)	0.005	26.2%/ 25.2%	90.4%/ 90.4%	0.603/ 0.611	ELISA
		47 (Cohort 2)	5	18	24	-	-	47 (Cohort 2)	0.032	27.7%/ 33.3%	91.5%/ 91.5%	0.628/ 0.636	ELISA
Ezrin	Li *et al*[62], 2017	149	4/14	57	71	3	149	98	< 0.0001	27.5%/ 27.8%	95.9%/ 95.9%	-	ELISA
STIP1	Xu *et al*[63], 2017	148 (Train-ing)	3/17	48	76	4	148	111 (Train-ing)	< 0.001	41.9%/ 35.7%	90.1%/ 90.1%	0.682/ 0.684	ELISA
		60 (Valida-tion)	1/8	20	30	1	60	40 (Valida-tion)	< 0.001	40.0%/ 38.5%	92.5%/ 92.5%	0.710/ 0.756	ELISA
Fascin	Chen *et al*[64], 2017	149	4/14	57	71	3	149	98	< 0.001	24.8%/ 20.6%	99.0%/ 99.0%	0.636/ 0.632	ELISA
DKK-1	Peng *et al*[65], 2016	185 (Train-ing)	4/23	69	85	4	185	97 (Train-ing)	< 0.0001	33.5%/ 34.6%	91.8%/ 91.8%	0.643/ 0.640	ELISA
		104 (Valida-tion)	1/12	35	53	3	104	53 (Valida-tion)	< 0.0001	33.7%/ 26.9%	92.5%/ 92.5%	0.629/ 0.603	ELISA
P16	Zhang *et al*[55], 2016	324 (Train-ing)	5/13	130	50	39	87	324 (Train-ing)	< 0.001	29.3%/-	81.8%/-	0.60/-	ELISA
		186 (Valida-tion)	1	29	14	46	96	186 (Valida-tion)	< 0.01	-	-	-	ELISA
	Jin *et al*[66], 2015	88	24	42	15	2	5	208	0.0052	5.7%/-	99.1%/-	-	ELISA
	Qin *et al*[58], 2014	174	3/8	79	52	18	-	242	< 0.05	18.4%/-	98.8%/-	0.6/-	ELISA
	Zhou *et al*[44], 2014	88	-	-	-	-	88	200	0.004	11.0%/-	97.0%/-	-	ELISA

	Looi *et al*[67], 2006	71	-	-	-	-	-	82	< 0.05	14.1%/-	98.8%/-	-	ELISA
p53	Zhang *et al*[55], 2016	324 (Training)	5/13	130	50	39	87	324 (Training)	< 0.001	55.9%/-	89.5%/-	0.784/-	ELISA
		186 (Validation)	1	29	14	46	96	186 (Validation)	< 0.001	-/-	-/-	-/-	ELISA
	Xu *et al*[43], 2014	388 (Test)	2/29	96	229	27	5	125 (Test)	< 0.0001	30.0%/-	98.0%/-	-	ELISA
		237 (Validation)	2/31	114	90	-	-	134 (Validation)	< 0.0001	29.0%/-	97.0%/-	-	ELISA
	Qin *et al*[58], 2014	174	3/8	79	52	18	-	242	< 0.05	21.8%/-	96.3%/-	0.6/-	ELISA
	Chai *et al*[68], 2014	157	-	-	-	-	157	85	< 0.01	22.9%/-	100%/-	-	ELISA
	Zhou *et al*[44], 2014	88	-	-	-	-	88	200	< 0.001	22.0%/-	98.0%/-	-	ELISA
	Cai *et al*[69], 2008	46	10	17	14	5	-	30	< 0.0001	39.1%/ 22.2%	100%/ 100%	-	ELISA
	Looi *et al*[67], 2006	71	-	-	-	-	71	82	< 0.05	7%/-	98.8%/-	-	ELISA
	Müller *et al*[70], 2006	50	-	-	-	-	50	436	< 0.05	20.0%/-	100%/-	-	Western blot
	Meglior-ino *et al*[59], 2005	77	-	-	-	-	77	82	< 0.01	14.3%/-	97.6%/-	-	ELISA
	Shimada *et al*[71], 2003	301	-	-	-	-	301	205	< 0.05	30.0%/-	95.5%/-	-	ELISA
	Shimada *et al*[72], 2002	105	50	24	21	10	-	153	< 0.001	26.7%/ 20.0%	95.5%/ 95.5%	-	ELISA
	Ralhan *et al*[73], 2000	60	-	-	-	-	60	50	< 0.05	60.0%/-	92.0%/-	-	ELISA
	Shimada *et al*[74], 2000	35	-	-	-	-	35	69	< 0.0001	40.0%/-	100.0%/-	-	ELISA
	Hagi-wara *et al*[75], 2000	46	6	15	24	2	-	13	< 0.05	28.0%/ 28.6%	100%/ 100%	-	ELISA
	Shimada *et al*[76], 1998	57	6/9	9	11	11	1	208	< 0.05	58.0%/-	99.0%/-	-	ELISA
	Sobti *et al*[77], 1998	20	-	-	-	-	20	20	0.0202	30.0%/-	100%/-	-	ELISA
	Cawley *et al*[78], 1988	23	-	-	-	-	23	19	0.0372	34.8%/-	94.7%/-	-	ELISA
NY-ESO-1	Xu *et al*[43], 2014	388 (Test)	2/29	96	229	27	5	125 (Test)	< 0.0001	26.0%/-	100%/-	-	ELISA
		237 (Validation)	2/31	114	90	-	-	134 (Validation)	< 0.0001	24.0%/-	99.0%/-	-	ELISA

	Oshima *et al*[79], 2016	172	-	-	-	-	172	74	< 0.001	32.0%/ 16.0%	100%/ 100%	-	ELISA
	Fujita *et al*[80], 2004	51	-	-	-	-	51	29	0.532	3.9%/-	100%/-	-	ELISA
P90	Zhang *et al*[55], 2016	324 (Training)	5/13	130	50	39	87	324 (Training)	< 0.001	31.5%/-	84.9%/-	0.617/-	ELISA
		186 (Validation)	1	29	14	46	96	186 (Validation)	< 0.001	-	-	-	ELISA
Mmp-7	Xu *et al*[43], 2014	388 (Test)	2/29	96	229	27	5	125 (Test)	< 0.001	9.0%/-	100%/-	-	ELISA
		237 (Validation)	2/31	114	90	-	-	134 (Validation)	< 0.001	10.0%/-	100%/-	-	ELISA
	Zhou *et al*[81], 2011	50	-	-	-	-	50	58	< 0.001	78.0%	81.0%	0.87/-	ELISA
Hsp70	Xu *et al*[43], 2014	388 (Test)	2/29	96	229	27	5	125 (Test)	< 0.001	11.0%/-	99.0%/-	-	ELISA
		237 (Validation)	2/31	114	90	-	-	134 (Validation)	< 0.01	8.0%/-	99.0%/-	-	ELISA
	Zhang *et al*[82], 2011	69	-	-	-	-	69	76	> 0.01	39.1%/-	92.3%/-	-	ELISA
	Fujita *et al*[40], 2008	16	2	7	4	3	-	13	< 0.001	93.7%/-	100%/-	-	ELISA
PRDX 6	Xu *et al*[43], 2014	388 (Test)	2/29	96	229	27	5	125 (Test)	< 0.001	11.0%/-	100%/-	-	ELISA
		237 (Validation)	2/31	114	90	-	-	134 (Validation)	< 0.001	10.0%/-	100%/-	-	ELISA
	Fujita *et al*[83], 2006	30	7	8	11	4	-	30	< 0.05	50.0%/ 53.5%	93.4%/ 93.4%	-	Western blot
Bmi-1	Xu *et al*[43], 2014	388 (Test)	2/29	96	229	27	5	125 (Test)	< 0.01	11.0%/-	98.0%/-	-	ELISA
		237 (Validation)	2/31	114	90	-	-	134 (Validation)	< 0.01	8.0%/-	100%/-	-	ELISA
	Liu *et al*[84], 2010	159	6	72	69	12	-	102	< 0.001	39.0%/-	100%/-	-	ELISA
Imp1	Zhang *et al*[55], 2016	324 (Training)	5/13	130	50	39	87	324 (Training)	< 0.001	26.9%/-	81.2%/-	0.576/-	ELISA
		186 (Validation)	1	29	14	46	96	186 (Validation)	< 0.01	-	-	-	ELISA
	Qin *et al*[58], 2014	174	3/8	79	52	18	-	242	< 0.05	16.1%/-	98.3%/-	0.6/-	ELISA
	Zhou *et al*[44], 2014	88	-	-	-	-	88	200	< 0.001	14.0%/-	99.0%/-	-	ELISA
Cyclin B1	Qin *et al*[58], 2014	174	3/8	79	52	18	-	242	< 0.05	16.1%/-	97.9%/-	0.6/-	ELISA
	Zhou *et al*[44], 2014	88	-	-	-	-	88	200	0.02	10.0%/-	97.0%/-	-	ELISA

C-Myc	Zhang *et al*[55], 2016	324 (Training)	5/13	130	50	39	87	324 (Training)	< 0.001	49.1%/-	81.5%/-	0.699/-	ELISA
		186 (Validation)	1	29	14	46	96	186 (Validation)	< 0.001	-	-	-	ELISA
	Qin *et al*[58], 2014	174	3/8	79	52	18	-	242	< 0.05	15.5%/-	98.8%/-	0.6/-	ELISA
	Zhou *et al*[44], 2014	88	-	-	-	-	88	200	< 0.001	18.0%/-	96.0%/-	-	ELISA
	Looi *et al*[67], 2006	71	-	-	-	-	-	82	< 0.05	7%/-	100%/-	-	ELISA
	Meglio-rino *et al*[59], 2005	77	-	-	-	-	77	82	< 0.01	11.7%/-	100%/-	-	ELISA
R^aI^A	Qin *et al*[58], 2014	174	3/8	79	52	18	-	242	< 0.05	15.5%/-	96.7%/-	0.6/-	ELISA
P62	Zhang *et al*[55], 2016	324 (Training)	5/13	130	50	39	87	324 (Training)	< 0.001	29.3%/-	81.8%/-	0.60/-	ELISA
		186 (Validation)	1	29	14	46	96	186 (Validation)	< 0.001	-	-	-	ELISA
	Qin *et al*[58], 2014	174	3/8	79	52	18	-	242	< 0.05	12.1%/-	95.9%/-	0.5/-	ELISA
	Zhou *et al*[44], 2014	88	-	-	-	-	88	200	0.001	13.0%/-	98.0%/-	-	ELISA
Koc	Zhang *et al*[55], 2016	324 (Training)	5/13	130	50	39	87	324 (Training)	< 0.001	35.8%/-	82.1%/-	0.63/-	ELISA
		186 (Validation)	1	29	14	46	96	186 (Validation)	< 0.05	-	-	-	ELISA
	Qin *et al*[58], 2014	174	3/8	79	52	18	-	242	< 0.05	11.5%/-	97.9%/-	0.5/-	ELISA
	Zhou *et al*[44], 2014	88	-	-	-	-	88	200	0.05	10.0%/-	96.0%/-	-	ELISA
Cyclin D1	Qin *et al*[58], 2014	174	3/8	79	52	18	-	242	< 0.05	10.3%/-	96.3%/-	0.5/-	ELISA
Cyclin E	Zhang *et al*[55], 2016	324 (Training)	5/13	130	50	39	87	324 (Training)	< 0.001	26.5%/-	83.0%/-	0.581/-	ELISA
		186 (Validation)	1	29	14	46	96	186 (Validation)	< 0.05	-	-	-	ELISA
	Qin *et al*[58], 2014	174	3/8	79	52	18	-	242	< 0.05	10.3%/-	99.2%/-	0.5/-	ELISA
HCCR	Zhang *et al*[55], 2016	324 (Training)	5/13	130	50	39	87	324 (Training)	< 0.001	34.6%/-	80.0%/-	0.596/-	ELISA
		186 (Validation)	1	29	14	46	96	186 (Validation)	< 0.001	-	-	-	ELISA
GSTO1	Li *et al*[85], 2014	67	-	-	-	-	67	90	< 0.01	44.8%/-	93.3%/-	-	ELISA
MDM2	Chai *et al*[68], 2014	157	-	-	-	-	157	85	< 0.01	14.0%/-	98.8%/-	-	ELISA

HSP105	Gao *et al*[51], 2014	46	7	-	-	-	39	40	< 0.01	39.1%/ 42.9%	95%/95%	0.794/-	Western blot
TIM	Gao *et al*[51], 2014	46	7	-	-	-	39	40	< 0.01	34.8%/ 28.6%	95%/95%	0.786/-	Western blot
Prdx1	Ren *et al*[86], 2013	68	-	-	-	-	68	89	< 0.01	13.2%/-	100%/-	-	ELISA, Western blot
FOXP3	Ye *et al*[87], 2013	97	26	45	19	2	5	227	< 0.0001	22.7%/-	95.2%/-	0.70/-	ELISA,
CD25	Guan *et al*[88], 2013	97	26	45	17	3	6	226	< 0.001	37.2%/-	90.0%/-	0.69/-	ELISA
ABCC3 (IgA)	Cheng *et al*[89], 2013	114	-	-	-	-	-	226	< 0.001	13.2%/-	>95%/-	0.65/-	ELISA
LY6K	Zhang *et al*[90], 2012	62	13		27	22	-	58	< 0.001	80.6%/ 73.2%	78.7%/ 78.7%	0.85/-	ELISA
HMGB1	Zhang *et al*[82], 2011	69	-	-	-	-	69	76	> 0.05	7.2%/-	98.7%/-	-	ELISA
ESCA-1	Kagaya *et al*[91], 2011	146	32	29	42	43	-	118	0.0001	21.2%/-	98.3%/-	-	ELISA
ESCA-2	Kagaya *et al*[91], 2011	72	37		35		72	98	0.0026	15.3%/ 8.1%	99.0%/ 99.0%	-	ELISA
ESCA-3	Kagaya *et al*[91], 2011	68	-	-	-	-	68	74	0.0079	16.2%/-	98.6%/-	-	ELISA
CDC25B	Dong *et al*[92], 2010	134	80		54		-	134	< 0.001	56.7%/-	91.0%	0.87/-	ELISA
	Liu *et al*[93], 2008	124	-	-	-	-	123	102	< 0.05	36.3%/-	100%/-	-	ELISA
GRP78	Tsunemi *et al*[94], 2010	15	-	-	-	-	15	20	< 0.05	26.7%/-	100%/-	-	Western blot
Makorin 1	Shimada *et al*[95], 2009	73	1/13	21	27	11	-	43	< 0.05	25.0%/ 22.9%	100%/ 100%	-	Western blot
CUEC-23	Shimada *et al*[96], 2009	54	7	13	18	16	-	46	< 0.05	26.0%/ 33.3%	96.0%/-	-	Western blot
	Shimada *et al*[96], 2009	29	1	11	8	9	-	46	0.036	17.0%/-	100%/-	-	ELISA
MMGL	Shimada *et al*[97], 2007	91	21	22	35	13	-	45	< 0.05	47.0%/ 38.0%	97.8%/ 97.8%	-	Western blot
TRIM21	Kubo-shima *et al*[98], 2006	91	39		52		-	42	< 0.05	20.0%/ 13.0%	100%/ 100%	-	Western blot
	Kubo-shima *et al*[98], 2006	54	-	-	-	-	54	42	0.013	15.0%/-	98.0%/-	-	ELISA
SLC2A1	Kubo-shima *et al*[99], 2006	57	19	6	13	19	-	31	< 0.001	21.0%/ 22.0%	100%/ 100%	-	ELISA
SURF1	Shimada *et al*[50], 2005	21	-	3	13	5	-	37	0.0003	48%/-	95%/-	-	ELISA

LOC 146223	Shimada *et al*[50], 2005	21	-	3	13	5	-	37	0.0028	38%/-	95%/-	-	ELISA
HOOK2	Shimada *et al*[50], 2005	21	-	3	13	5	-	37	0.0431	14%/-	100%/-	-	ELISA
AGENCOURT_7565913	Shimada *et al*[50], 2005	21	-	3	13	5	-	37	0.0431	14%/-	100%/-	-	ELISA
TROP2	Nakashima *et al*[100], 2004	75	14	14	24	23	-	43	< 0.05	31.0%/21.0%	97.7%/97.7%	-	Western blot

ESCC: Esophageal squamous cell carcinoma; AUC: Area under the curve; L1CAM: L1-cell adhesion molecule; STIP1: Stress induced phosphoprotein 1; DKK-1: Dickkopf 1; Mmp-7: Matrix metallopeptidase 7; Hsp70: Heat shock protein 70; PRDX 6: Peroxiredoxin 6; Bmi-1: BMI1 proto-oncogene, polycomb ring finger; Imp1: Insulin-like growth factor 2 mRNA binding protein 1; C-Myc: MYC proto-oncogene, bHLH transcription factor; Koc: Insulin-like growth factor 2 mRNA binding protein 3; HCCR: LETM1 domain containing 1; GSTO1: Glutathione S-transferase omega 1; MDM2: MDM2 proto-oncogene; HSP105: Heat shock protein family H (Hsp110) member 1; TIM: Rho guanine nucleotide exchange factor 5; Prdx1: Peroxiredoxin 1; FOXP3: Forkhead box P3; CD25: Interleukin 2 receptor subunit alpha; ABCC3: ATP binding cassette subfamily C member 3; LY6K: Lymphocyte antigen 6 family member K; HMGB1, high mobility group box 1; CDC25B: Cell division cycle 25B; GRP78: Heat shock protein family A (Hsp70) member 5; Makorin 1: Makorin ring finger protein 1; MMGL: Myomegalin; TRIM21: Tripartite motif containing 21; SLC2A1: Solute carrier family 2 member 1; SURF1: SURF1 cytochrome c oxidase assembly factor; HOOK2: Hook microtubule tethering protein 2; TROP2: Tumor-associated calcium signal transducer 2.

DIAGNOSTIC PERFORMANCE OF AUTOANTIBODY PANELS IN ESCC

Over the past few years, as single TA autoantibodies do not appear to demonstrate enough diagnostic sensitivity to set up a reliable test for early detection, studies have aimed to identify a suitable panel of TA autoantibodies. These predicaments are presumably due to cancer heterogeneity. In fact, it is unlikely that most patients will respond to the same immunodominant antigens. Even tumors of the same kind are comprised of a diverse mix of biological subtypes; accordingly, cancer patients are more likely to induce an immunoreaction to different sets of TAA, and not all cancers are likely to be detected by autoantibodies against a single antigen. Tables 4 and 5 give an overview of different combinations of multiple autoantibodies as potential blood-based biomarkers for ESCC and EGJA that have been described in the literature by various research groups.

With improvements in technology, several high-throughput methods, such as proteomics platforms, have enabled the uncovering of autoantibodies and the generation of a panel of TAA. These discovery techniques encompass serological analysis of tumor antigens by recombinant cDNA expression cloning[45], serological proteome analysis[46], phage display[47], protein microarrays[48] and multiple affinity protein profiling[49]. Shimada *et al*[50] were the first to use the high-throughput method of serological analysis of tumor antigens by recombinant cDNA expression cloning in ESCC. They showed that several TAA that could elicit a humoral immune response that could be detected simultaneously, and the technique enabled the generation of an autoantibody panel that exhibited better diagnostic value (86% sensitivity and 100% specificity) than a single TA autoantibody. Subsequently, a study using serological proteome analysis identified some novel TAA associated with ESCC, and the combination of two TAA (HSP105 and TIM) can give 54.3% sensitivity and 95% specificity in distinguishing ESCC from controls[51]. These studies all show that the combined detection of autoantibodies against several antigens in the panel can greatly increase sensitivity in the diagnosis of ESCC. However, except for the two above-mentioned studies, no other relevant literature applying proteomic technology to identify a TAA panel has appeared. This indicates, to some extent, that the identification and development of novel autoantibodies by proteomics platforms for ESCC is limited and behindhand especially compared with other tumor types, such as lung, breast and liver tumors[52-54].

On the other hand, researchers have been more inclined to evaluate the diagnostic performance of combinations of several known TAA. In accord with such thinking, eight studies reported the diagnostic value of different combinations of autoantibodies for ESCC (Table 4). From the list of autoantibodies examined in the panel (Table 4), p53 autoantibodies were the most common choice for inclusion in the biomarker combinations. As is known, p53 as a tumor suppressor gene has been linked to many cancers, including ESCC, and thus would be a rational biomarker to be investigated. Zhang *et al*[55] assessed a combination of six immunoreactive TAA in ESCC samples and normal controls with independent validation. Then, they sought to

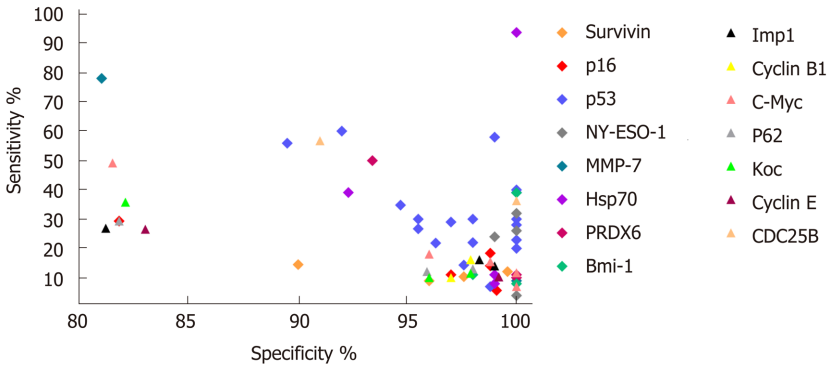

Figure 1 Graphical representation of sensitivity *versus* specificity for single tumor-associated autoantibody biomarkers in esophageal squamous cell carcinoma reported in more than one study. Mmp-7: Matrix metallopeptidase 7; Hsp70: Heat shock protein 70; PRDX 6: Peroxiredoxin 6; Bmi-1: BMI1 proto-oncogene, polycomb ring finger; Koc: Insulin-like growth factor 2 mRNA binding protein 3; C-Myc: MYC proto-oncogene, bHLH transcription factor; IMP1: Insulin-like growth factor 2 mRNA binding protein 1.

identify which biomarkers used in combination were more informative and allowed a similar discrimination between groups. They finally found a restricted panel of four TAA that gave similar sensitivity and specificity in early stage ESCC. Indeed, a similar research strategy had been previously performed by Xu *et al*[43] who used two independent cohorts to investigate the combination of autoantibodies against p53, NY-ESO-1, MMP-7, Hsp70, Prx VI and Bmi-1. This panel distinguished early stage ESCC from normal controls with a sensitivity/specificity of 45%/95% and 46%/96%, respectively in the test and validation cohorts. Interestingly, the authors also determined a simplified autoantibody panel retaining four out of six biomarkers that exhibited almost the same diagnostic efficacy (Table 4). Although it is reported that a majority of biomarkers with desirable outcomes in a first data set often result in less promising results in additional independent data sets[56], the two above studies with the combinations of known TAA all showed satisfactory diagnostic value in independent validation cohorts. This suggests potential clinical applications for autoantibody combinations to diagnose ESCC. However, we can see from Table 4 that most of the studies reviewed lack validation in an independent population. In practice, the results of biomarkers need to be validated in larger multicenter cohorts and evaluated as a screening test in high-risk populations. However, no study on the evaluation of autoantibodies in ESCC diagnosis has been able to do so. All previously identified autoantibody panels for ESCC should be validated by these procedures to evaluate their true clinical relevance and diagnostic power.

DIAGNOSTIC PERFORMANCE OF AUTOANTIBODY PANELS IN EGJA

As the combined detection of selected autoantibodies as a panel could generally increase diagnostic sensitivity while keeping relatively high specificity in ESCC, two studies have attempted to evaluate the same panels of autoantibodies identified in ESCC for early detection of EGJA and have shown promising results. They demonstrated sensitivities above 50% and specificities above 86% (Table 5). Zhou *et al*[41] detected autoantibodies to eight TAA, comprised of p53, IMP1, P16, cyclin B1, P62, c-Myc, survivin and Koc and suggested that successive addition of seven TAA (p53, Koc, P62, c-Myc, IMP1, survivin and P16) led to stepwise increases in sensitivity and specificity, ultimately achieving a sensitivity of 64.0% with a specificity of 87.0%. This optimized combination is somewhat different from an optimized panel identified for ESCC (p53, IMP1, P16, cyclin B1, P62 and c-Myc) studied by the same research team. Subsequently, Xu *et al*[42] showed that autoantibodies against a combination of p53, NY-ESO-1, MMP-7, Hsp70, PRDX6 and Bmi-1, which is the same as the panel used for evaluation of ESCC, could be potentially used for early diagnosis of EGJA. When comparing stage I and II patients to normal controls, the authors showed sensitivities and specificities of 50.0% and 90.5% and 56.0% and 90.0%, respectively, in the training and validation cohorts. It should be noted that a strict panel of p53, NY-ESO-1 and Bmi-1 to comprise informative biomarkers for EGJA gives similar diagnostic performance. Interestingly, as discussed above, a different restricted combination (p53, NY-ESO-1, PRDX6 and Hsp70) from the same autoantibody panel

Table 3 Diagnostic performance of single tumor-associated autoantibody biomarkers in esophagogastric junction adenocarcinoma

Target antigen of auto-anti-bodies	Authors, year	EGJA cases, *n*	Stage, *n*					Con-trols, *n*	*P* value	Sensiti-vity, all stages/ early stage	Specifi-city, all stages/ early stage	AUC, all stages/ early stage	Method
			I	II	III	IV	Tx						
p53	Xu *et al*[42], 2019	122 (Train-ing)	2	16	87	17	122	169 (Valida-tion)	< 0.0001	35.2%/ 33.3%	90.5%/ 90.5%	0.718/ 0.648	ELISA
		70 (Train-ing)	11	14	30	15	80	80 (Valida-tion)	< 0.0001	35.7%/ 40.0%	96.3%/ 96.3%	0.766/ 0.799	ELISA
	Zhou *et al*[41], 2015	75	-	-	-	-	75	140	< 0.001	24.0%/-	92%/-	0.67/-	ELISA
NY-ESO-1	Xu *et al*[42], 2019	122 (Train-ing)	2	16	87	17	122	169 (Valida-tion)	< 0.0001	37.7%/ 27.8%	90.5%/ 90.5%	0.718/ 0.654	ELISA
		70 (Train-ing)	11	14	30	15	80	80 (Valida-tion)	< 0.0001	34.3%/ 28.0%	95.0%/ 95.0%	0.747/ 0.714	ELISA
PRDX6	Xu *et al*[42], 2019	122 (Train-ing)	2	16	87	17	122	169 (Valida-tion)	0.033	34.4%/ 38.9%	90.5%/ 90.5%	0.573/ 0.602	ELISA
		70 (Train-ing)	11	14	30	15	80	80 (Valida-tion)	0.002	30.0%/ 28.0%	90.0%/ 90.0%	0.647/ 0.629	ELISA
MMP-7	Xu *et al*[42], 2019	122 (Train-ing)	2	16	87	17	122	169 (Valida-tion)	0.005	30.3%/ 33.3%	90.5%/ 90.5%	0.597/ 0.575	ELISA
		70 (Train-ing)	11	14	30	15	80	80 (Valida-tion)	0.036	24.3%/ 28.0%	95.0%/ 95.0%	0.599/ 0.609	ELISA
Hsp70	Xu *et al*[42], 2019	122 (Train-ing)	2	16	87	17	122	169 (Valida-tion)	< 0.0001	18.0%/ 16.7%	90.5%/ 90.5%	0.652/ 0.697	ELISA
		70 (Train-ing)	11	14	30	15	80	80 (Valida-tion)	< 0.0001	28.6%/ 32.0%	86.3%/ 86.3%	0.686/ 0.702	ELISA
Bmi-1	Xu *et al*[42], 2019	122 (Train-ing)	2	16	87	17	122	169 (Valida-tion)	< 0.0001	22.1%/ 27.8%	90.5%/ 90.5%	0.686/ 0.685	ELISA
		70 (Train-ing)	11	14	30	15	80	80 (Valida-tion)	< 0.0001	54.3%/ 40.0%	90.0%/ 90.0%	0.711/ 0.682	ELISA
Koc	Zhou *et al*[41], 2015	75	-	-	-	-	75	140	0.05	19.0%/-	91%/-	-	ELISA
P62	Zhou *et al*[41], 2015	75	-	-	-	-	75	140	0.02	16.0%/-	94%/-	-	ELISA
C-Myc	Zhou *et al*[41], 2015	75	-	-	-	-	75	140	0.18	11.0%/-	94%/-	-	ELISA
IMP1	Zhou *et al*[41], 2015	75	-	-	-	-	75	140	0.04	13.0%/-	95%/-	-	ELISA
Survivin	Zhou *et al*[41], 2015	75	-	-	-	-	75	140	0.002	17.0%/-	96%/-	-	ELISA
P16	Zhou *et al*[41], 2015	75	-	-	-	-	75	140	0.01	15.0%/-	96%/-	-	ELISA
Cyclin B1	Zhou *et al*[41], 2015	75	-	-	-	-	75	140	0.01	12.0%/-	97%/-	-	ELISA

EGJA: Esophagogastric junction adenocarcinoma; AUC: Area under the curve; PRDX 6: Peroxiredoxin 6; Mmp-7: Matrix metallopeptidase 7; Hsp70: Heat shock protein 70; Bmi-1: BMI1 proto-oncogene, polycomb ring finger; Koc: Insulin-like growth factor 2 mRNA binding protein 3; C-Myc: MYC proto-oncogene, bHLH transcription factor; IMP1: Insulin-like growth factor 2 mRNA binding protein 1.

in early stage ESCC retains high sensitivity and specificity.

These studies suggest that the importance of individual autoantibodies in the panel assay varies in different types of cancers. However, we still need to determine which TA autoantibodies applied in combination are more informative and allow a better diagnostic value. In future work, more TA autoantibodies need to be discovered and characterized to identify the best combination for EGJA. Meanwhile, the identified signatures for EGJA should be verified in larger multicenter-appropriated cohorts of early stage patients and controls to test the diagnostic power.

CONCLUSION AND PERSPECTIVES

Endoscopic examination is a current but invasive diagnostic and screening procedure for early detection of ESCC and EGJA. The development and validation of non-invasive biomarkers is of great need for ESCC and EGJA screening. In recent decades, a large number of blood-based cancer biomarkers, such as cell-free circulating tumor DNAs, various non-coding RNAs, proteins and TA autoantibodies, have been identified and indicate the potential for early detection of esophageal cancer. Among these biomarkers, TA autoantibodies are a promising biomarker entity in early cancer detection as they are capable of identifying cancer in high-risk individuals. Moreover, they are highly stable and can be easily detected by routine methods (*e.g.*, ELISA). Recently, a TA autoantibody assay named *EarlyCDT*-Lung (against p53, NY-ESO-1, CAGE, GBU4-5, MAGE A4, SOX2 and Hu-D) approved by the FDA has been clinically and analytically validated. An ongoing prospective randomized trial is evaluating the clinical utility of this TA autoantibody panel and its use in a clinical setting of which the results are expected to be announced in the near future. Once this assay is successful for lung cancer, we would predict that tests for all solid tumors, including ESCC and EGJA, will follow.

Biomarker development needs several gradual steps covering preclinical studies, retrospective studies of stored specimens, multicenter validation studies and prospective screening studies. However, in ESCC and EGJA, autoantibody studies on early detection are hampered by several issues. First, the availability of sera from early stage patients seems limited. Only few studies have investigated the diagnostic value of TA autoantibody panels in patients with early stage tumors. Access to large early stage sample cohorts is an essential and necessary issue to examine a test's value for early stage disease. Moreover, few patients with pre-diagnostic serum samples or high-risk ESCC or EGJA cohorts are available, and up to now no study has reported on the immune response in the form of autoantibodies in these populations. Thus, an investigation of TA autoantibodies for the early detection of ESCC and EGJA will be limited mainly by the availability of human samples. On the other hand, current studies (Tables 4 and 5) investigating autoantibodies show promise but still lack the necessary validation stages. These studies need clinical multicenter validation through use of a broader population to further determine diagnostic value.

It seems that there are different patterns of TA autoantibody frequencies in different types of cancers. Thus, one encountered difficulty is the definition of the panel. This leads to the question of how to choose the optimized combination that works best in terms of sensitivity, specificity and predictive value. At this moment, these is no good guiding principle, but more advanced high-throughput proteome technology might be helpful. On the other hand, it should also be pointed out that TA autoantibodies may not be unique for specific types of cancers. Therefore, TA autoantibody panels identified for ESCC or EGJA are likely to be used as a screening test to discover the existence of cancer, and in general more specific diagnostic tools, such as endoscopy, should be carried out in the event of a positive result.

In conclusion, this review suggests that TA autoantibodies have the potential to serve as diagnostic biomarkers for ESCC and EGJA possibly as part of a general cancer screen. However, present studies in ESCC and EGJA remain at an early stage. It is clear that extensive efforts are needed to uncover promising autoantibody signatures to detect these cancers especially at the early stage. Moreover, it is too early to evaluate the diagnostic value of the autoantibodies reviewed here for clinical use. Standardized assay protocols facilitating the establishment of autoantibodies as highly accurate biomarkers is of great need in ESCC and EGJA. Finally, future studies performed with precise design and collaborative efforts among groups to build

Table 4 Diagnostic performance of tumor-associated autoantibody panel in esophageal squamous cell carcinoma

Target antigen of auto-anti-bodies	Authors, year	ESCC cases, *n*	Stage, *n*					Con-trols, *n*	Sensiti-vity, all stages/ early stage	Specifi-city, all stages/ early stage	AUC, all stages/ early stage	Method
			0/I	II	III	IV	Unknown					
c-Myc, HCCR, IMP1, Koc, p53 and p62	Zhang *et al*[55], 2016	324 (Train-ing)	5/13	130	50	39	87	324 (Train-ing)	67.9%/ 66.9%	86.7%/ 86.7%	0.838/ 0.829	ELISA
		186 (Valida-tion)	1	29	14	46	96	186 (Valida-tion)	67.7%/ 56.7%	85.5%/ 85.5%	0.859/ 0.818	ELISA
c-Myc, HCCR, p53 and p62	Zhang *et al*[55], 2016	324 (Train-ing)	5/13	130	50	39	87	324 (Train-ing)	67.6%/ 67.6%	86.4%/ 86.4%	0.838/ 0.831	ELISA
		186 (Valida-tion)	1	29	14	46	96	186 (Valida-tion)	72.0%/ 63.3%	85.0%/ 85.0%	0.872/ 0.837	ELISA
MAGEA 4, CTAG1, TP53, SDCCA G8 and ERBB2_C	Werner *et al*[101], 2016	31	-	-	-	-	31	321	26.0%/-	88.5%/-	-	Bead-based multiplex serology
p53 and MDM2	Chai *et al*[68], 2014	157	-	-	-	-	157	85	35.0%/-	98.8%/-	-	ELISA
p53, pl6, Imp-l, CyclinB1 , c-Myc, RalA, p62, Survivin, Koc, Cyclin D1 and Cyclin E	Qin *et al*[58], 2014	174	3/8	79	52	18	-	242	75.3%/-	81.0%/-	0.78/-	ELISA
p53, NY-ESO-1, MMP-7, Hsp70, PRDX 6 and Bmi-1	Xu *et al*[43], 2014	388 (Test)	2/29	96	229	27	5	125 (Test)	57.0%/ 45.0%	95.0%/ 95.0%	-	ELISA
		237 (Valida-tion)	2/31	114	90	-	-	134 (Valida-tion)	51.0%/ 46.0%	96.0%/ 96.0%	-	ELISA
p53, NY-ESO-1, Hsp70 and PRDX 6	Xu *et al*[43], 2014	388 (Test)	2/29	96	229	27	5	125 (Test)	55.0%/ 45.0%	98.0%/ 98.0%	-	ELISA
		237 (Valida-tion)	2/31	114	90	-	-	134 (Valida-tion)	48.0%/ 45.0%	96.0%/ 96.0%	-	ELISA
p53, IMP1, P16, Cyclin B1, P62, and C-myc	Zhou *et al*[44], 2014	88	-	-	-	-	88	200	64.0%/-	94.0%/-	0.78/-	ELISA
HSP105 and TIM	Gao *et al*[51], 2014	46	7	-	-	-	39	40	54.3%/-	95.0%/-	0.823/-	Western blot

p16, c-Myc and p53	Looi *et al*[67], 2006	71	-	-	-	-	71	82	7%/-	100%/-	-	ELISA
SURF1, LOC146223, HOOK2 and AGENCOURT_7565913	Shimada *et al*[50], 2005	21	-	3	13	5	-	37	86%/-	100%/-	-	ELISA
Survivin, p53 and C-myc	Meglio-rino *et al*[59], 2005	77	-	-	-	-	77	82	29.9%/-	95.1%/-	-	ELISA

ESCC: Esophageal squamous cell carcinoma; AUC: Area under the curve; C-Myc: MYC proto-oncogene, bHLH transcription factor; HCCR: LETM1 domain containing 1; IMP1: Insulin-like growth factor 2 mRNA binding protein 1; Koc: Insulin-like growth factor 2 mRNA binding protein 3; MAGEA4: MAGE family member A4; CTAG1: Cancer/testis antigen 1B; SDCCAG8: Serologically defined colon cancer antigen 8; ERBB2: Erb-b2 receptor tyrosine kinase 2; MDM2: MDM2 proto-oncogene; HSP105: Heat shock protein family H (Hsp110) member 1; TIM: Rho guanine nucleotide exchange factor 5; SURF1: SURF1 cytochrome c oxidase assembly factor; HOOK2: Hook microtubule tethering protein 2.

standardized guidelines to report results will contribute greatly in this research area.

Table 5 Diagnostic performance of tumor-associated autoantibody panel in esophagogastric junction adenocarcinoma

Target antigen of auto-anti-bodies	Authors, year	EGJA cases, *n*	Stage, *n*					Con-trols, *n*	Sensiti-vity, all stages/ early stage	Specifi-city, all stages/ early stage	AUC, all stages/ early stage	Method
			0/I	II	III	IV	Unknown					
p53, NY-ESO-1, MMP-7, Hsp70, PRDX6 and Bmi-1	Xu *et al*[42], 2019	122 (Train-ing)	2	16	87	17	122	169 (Valida-tion)	59.0%/ 50.0%	90.5%/ 90.5%	0.818/ 0.786	ELISA
		70 (Valida-tion)	11	14	30	15	80	80 (Valida-tion)	61.4%/ 56.0%	90.0%/ 90.0%	0.815/ 0.786	ELISA
p53, NY-ESO-1 and Bmi-1	Xu *et al*[42], 2019	122 (Train-ing)	2	16	87	17	122	169 (Valida-tion)	53.5%/ 55.6%	90.5%/ 90.5%	0.814/ 0.744	ELISA
		70 (Valida-tion)	11	14	30	15	80	80 (Valida-tion)	60.0%/ 52.0%	93.7%/ 93.7%	0.823/ 0.773	ELISA
p53, Koc, P62, c-Myc, IMP1, Survivin and P16	Zhou *et al*[41], 2015	75	-	-	-	-	75	140	64.0%/-	87.0%/-	0.73/-	ELISA

EGJA: Esophagogastric junction adenocarcinoma; AUC: Area under the curve; Mmp-7: Matrix metallopeptidase 7; Hsp70: Heat shock protein 70; PRDX 6: Peroxiredoxin 6; Bmi-1: BMI1 proto-oncogene, polycomb ring finger; Koc: Insulin-like growth factor 2 mRNA binding protein 3; C-Myc: MYC proto-oncogene, bHLH transcription factor; IMP1: Insulin-like growth factor 2 mRNA binding protein 1.

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REFERENCES

1 **Bray F**, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin* 2018; **68**: 394-424 [PMID: 30207593 DOI: 10.3322/caac.21492]
2 **Colquhoun A**, Arnold M, Ferlay J, Goodman KJ, Forman D, Soerjomataram I. Global patterns of cardia and non-cardia gastric cancer incidence in 2012. *Gut* 2015; **64**: 1881-1888 [PMID: 25748648 DOI: 10.1136/gutjnl-2014-308915]
3 **Devesa SS**, Blot WJ, Fraumeni JF. Changing patterns in the incidence of esophageal and gastric carcinoma in the United States. *Cancer* 1998; **83**: 2049-2053 [PMID: 9827707 DOI: 10.1002/(SICI)1097-0142(19981115)83:10<2049::AID-CNCR1>3.0.CO;2-2]
4 **Zhou Y**, Zhang Z, Zhang Z, Wu J, Ren D, Yan X, Wang Q, Wang Y, Wang H, Zhang J, Zhu X, Yang Y, Luo C, Guo X, Tang C, Qiao L. A rising trend of gastric cardia cancer in Gansu Province of China. *Cancer Lett* 2008; **269**: 18-25 [PMID: 18501504 DOI: 10.1016/j.canlet.2008.04.013]
5 **Tran GD**, Sun XD, Abnet CC, Fan JH, Dawsey SM, Dong ZW, Mark SD, Qiao YL, Taylor PR. Prospective study of risk factors for esophageal and gastric cancers in the Linxian general population trial cohort in China. *Int J Cancer* 2005; **113**: 456-463 [PMID: 15455378 DOI: 10.1002/ijc.20616]
6 **Chen H**, Wang LD, Guo M, Gao SG, Guo HQ, Fan ZM, Li JL. Alterations of p53 and PCNA in cancer and adjacent tissues from concurrent carcinomas of the esophagus and gastric cardia in the same patient in Linzhou, a high incidence area for esophageal cancer in northern China. *World J Gastroenterol* 2003; **9**: 16-21 [PMID: 12508343 DOI: 10.3748/wjg.v9.i1.16]
7 **Enzinger PC**, Mayer RJ. Esophageal cancer. *N Engl J Med* 2003; **349**: 2241-2252 [PMID: 14657432 DOI: 10.1056/NEJMra035010]
8 **Kim T**, Grobmyer SR, Smith R, Ben-David K, Ang D, Vogel SB, Hochwald SN. Esophageal cancer--the five year survivors. *J Surg Oncol* 2011; **103**: 179-183 [PMID: 21259254 DOI: 10.1002/jso.21784]
9 **Wang GQ**, Jiao GG, Chang FB, Fang WH, Song JX, Lu N, Lin DM, Xie YQ, Yang L. Long-term results of operation for 420 patients with early squamous cell esophageal carcinoma discovered by screening. *Ann Thorac Surg* 2004; **77**: 1740-1744 [PMID: 15111177 DOI: 10.1016/j.athoracsur.2003.10.098]

10 **Pech O**, Behrens A, May A, Nachbar L, Gossner L, Rabenstein T, Manner H, Guenter E, Huijsmans J, Vieth M, Stolte M, Ell C. Long-term results and risk factor analysis for recurrence after curative endoscopic therapy in 349 patients with high-grade intraepithelial neoplasia and mucosal adenocarcinoma in Barrett's oesophagus. *Gut* 2008; **57**: 1200-1206 [PMID: 18460553 DOI: 10.1136/gut.2007.142539]

11 **Chapman CJ**, Thorpe AJ, Murray A, Parsy-Kowalska CB, Allen J, Stafford KM, Chauhan AS, Kite TA, Maddison P, Robertson JF. Immunobiomarkers in small cell lung cancer: Potential early cancer signals. *Clin Cancer Res* 2011; **17**: 1474-1480 [PMID: 21138858 DOI: 10.1158/1078-0432.CCR-10-1363]

12 **Zhong L**, Coe SP, Stromberg AJ, Khattar NH, Jett JR, Hirschowitz EA. Profiling tumor-associated antibodies for early detection of non-small cell lung cancer. *J Thorac Oncol* 2006; **1**: 513-519 [PMID: 17409910 DOI: 10.1016/S1556-0864(15)30352-X]

13 **Trivers GE**, De Benedetti VM, Cawley HL, Caron G, Harrington AM, Bennett WP, Jett JR, Colby TV, Tazelaar H, Pairolero P, Miller RD, Harris CC. Anti-p53 antibodies in sera from patients with chronic obstructive pulmonary disease can predate a diagnosis of cancer. *Clin Cancer Res* 1996; **2**: 1767-1775 [PMID: 9816128 DOI: 10.1093/carcin/17.10.2275]

14 **Takeda A**, Shimada H, Nakajima K, Imaseki H, Suzuki T, Asano T, Ochiai T, Isono K. Monitoring of p53 autoantibodies after resection of colorectal cancer: Relationship to operative curability. *Eur J Surg* 2001; **167**: 50-53 [PMID: 11213822 DOI: 10.1080/110241501750069828]

15 **Anderson KS**, Wong J, Vitonis A, Crum CP, Sluss PM, Labaer J, Cramer D. p53 autoantibodies as potential detection and prognostic biomarkers in serous ovarian cancer. *Cancer Epidemiol Biomarkers Prev* 2010; **19**: 859-868 [PMID: 20200435 DOI: 10.1158/1055-9965.EPI-09-0880]

16 **Shan Q**, Lou X, Xiao T, Zhang J, Sun H, Gao Y, Cheng S, Wu L, Xu N, Liu S. A cancer/testis antigen microarray to screen autoantibody biomarkers of non-small cell lung cancer. *Cancer Lett* 2013; **328**: 160-167 [PMID: 22922091 DOI: 10.1016/j.canlet.2012.08.019]

17 **Fosså A**, Berner A, Fosså SD, Hernes E, Gaudernack G, Smeland EB. NY-ESO-1 protein expression and humoral immune responses in prostate cancer. *Prostate* 2004; **59**: 440-447 [PMID: 15065093 DOI: 10.1002/pros.20025]

18 **Jäger E**, Stockert E, Zidianakis Z, Chen YT, Karbach J, Jäger D, Arand M, Ritter G, Old LJ, Knuth A. Humoral immune responses of cancer patients against "Cancer-Testis" antigen NY-ESO-1: Correlation with clinical events. *Int J Cancer* 1999; **84**: 506-510 [PMID: 10502728 DOI: 10.1002/(sici)1097-0215(19991022)84:5<506::aid-ijc10>3.0.co;2-6]

19 **Pedersen JW**, Gentry-Maharaj A, Nøstdal A, Fourkala EO, Dawnay A, Burnell M, Zaikin A, Burchell J, Papadimitriou JT, Clausen H, Jacobs I, Menon U, Wandall HH. Cancer-associated autoantibodies to MUC1 and MUC4--a blinded case–control study of colorectal cancer in UK collaborative trial of ovarian cancer screening. *Int J Cancer* 2014; **134**: 2180-2188 [PMID: 24122770 DOI: 10.1002/ijc.28538]

20 **Kurtenkov O**, Klaamas K, Mensdorff-Pouilly S, Miljukhina L, Shljapnikova L, Chuzmarov V. Humoral immune response to MUC1 and to the Thomsen-Friedenreich (TF) glycotope in patients with gastric cancer: Relation to survival. *Acta Oncol* 2007; **46**: 316-323 [PMID: 17450466 DOI: 10.1080/02841860601055441]

21 **Graus F**, Dalmou J, Reñé R, Tora M, Malats N, Verschuuren JJ, Cardenal F, Viñolas N, Garcia del Muro J, Vadell C, Mason WP, Rosell R, Posner JB, Real FX. Anti-Hu antibodies in patients with small-cell lung cancer: Association with complete response to therapy and improved survival. *J Clin Oncol* 1997; **15**: 2866-2872 [PMID: 9256130 DOI: 10.1200/jco.1997.15.8.2866]

22 **Baldwin RW**. Tumour-specific immunity against spontaneous rat tumours. *Int J Cancer* 1966; **1**: 257-264 [PMID: 5944065 DOI: 10.1002/ijc.2910010305]

23 **Baldwin RW**. An immunological approach to cancer. *Lav Ist Anat Istol Patol Univ Studi Perugia* 1968; **28**: 65-85 [PMID: 4882305]

24 **Baldwin RW**. Tumour-associated antigens and tumour-host interactions. *Proc R Soc Med* 1971; **64**: 1039-1042 [PMID: 4335921]

25 **Dunn GP**, Bruce AT, Ikeda H, Old LJ, Schreiber RD. Cancer immunoediting: From immunosurveillance to tumor escape. *Nat Immunol* 2002; **3**: 991-998 [PMID: 12407406 DOI: 10.1038/ni1102-991]

26 **Schreiber RD**, Old LJ, Smyth MJ. Cancer immunoediting: Integrating immunity's roles in cancer suppression and promotion. *Science* 2011; **331**: 1565-1570 [PMID: 21436444 DOI: 10.1126/science.1203486]

27 **Vivier E**, Ugolini S, Blaise D, Chabannon C, Brossay L. Targeting natural killer cells and natural killer T cells in cancer. *Nat Rev Immunol* 2012; **12**: 239-252 [PMID: 22437937 DOI: 10.1038/nri3174]

28 **Finn OJ**. Immuno-oncology: Understanding the function and dysfunction of the immune system in cancer. *Ann Oncol* 2012; **23** Suppl 8: viii6-viii9 [PMID: 22918931 DOI: 10.1093/annonc/mds256]

29 **Kim R**, Emi M, Tanabe K, Arihiro K. Tumor-driven evolution of immunosuppressive networks during malignant progression. *Cancer Res* 2006; **66**: 5527-5536 [PMID: 16740684 DOI: 10.1158/0008-5472.can-05-4128]

30 **Winter SF**, Minna JD, Johnson BE, Takahashi T, Gazdar AF, Carbone DP. Development of antibodies against p53 in lung cancer patients appears to be dependent on the type of p53 mutation. *Cancer Res* 1992; **52**: 4168-4174 [PMID: 1322237 DOI: 10.1046/j.1365-2109.2002.00715.x]

31 **Pardoll D**. Does the immune system see tumors as foreign or self? *Annu Rev Immunol* 2003; **21**: 807-839 [PMID: 12615893 DOI: 10.1146/annurev.immunol.21.120601.141135]

32 **Chen YT**, Scanlan MJ, Sahin U, Türeci O, Gure AO, Tsang S, Williamson B, Stockert E, Pfreundschuh M, Old LJ. A testicular antigen aberrantly expressed in human cancers detected by autologous antibody screening. *Proc Natl Acad Sci U S A* 1997; **94**: 1914-1918 [PMID: 9050879 DOI: 10.1073/pnas.94.5.1914]

33 **Goodell V**, Waisman J, Salazar LG, de la Rosa C, Link J, Coveler AL, Childs JS, Fintak PA, Higgins DM, Disis ML. Level of HER-2/neu protein expression in breast cancer may affect the development of endogenous HER-2/neu-specific immunity. *Mol Cancer Ther* 2008; **7**: 449-454 [PMID: 18319334 DOI: 10.1158/1535-7163.MCT-07-0386]

34 **Plotz PH**. The autoantibody repertoire: Searching for order. *Nat Rev Immunol* 2003; **3**: 73-78 [PMID: 12511877 DOI: 10.1038/nri976]

35 **Burford B**, Gentry-Maharaj A, Graham R, Allen D, Pedersen JW, Nudelman AS, Blixt O, Fourkala EO, Bueti D, Dawnay A, Ford J, Desai R, David L, Trinder P, Acres B, Schwientek T, Gammerman A, Reis CA, Silva L, Osório H, Hallett R, Wandall HH, Mandel U, Hollingsworth MA, Jacobs I, Fentiman I, Clausen H, Taylor-Papadimitriou J, Menon U, Burchell JM. Autoantibodies to MUC1 glycopeptides cannot be used as a screening assay for early detection of breast, ovarian, lung or pancreatic cancer. *Br J Cancer* 2013; **108**: 2045-2055 [PMID: 23652307 DOI: 10.1038/bjc.2013.214]

36 **Reuschenbach M**, von Knebel Doeberitz M, Wentzensen N. A systematic review of humoral immune

responses against tumor antigens. *Cancer Immunol Immunother* 2009; **58**: 1535-1544 [PMID: 19562338 DOI: 10.1007/s00262-009-0733-4]

37 **Matsumoto I**, Maccioni M, Lee DM, Maurice M, Simmons B, Brenner M, Mathis D, Benoist C. How antibodies to a ubiquitous cytoplasmic enzyme may provoke joint-specific autoimmune disease. *Nat Immunol* 2002; **3**: 360-365 [PMID: 11896391 DOI: 10.1038/ni772]

38 **Binstadt BA**, Patel PR, Alencar H, Nigrovic PA, Lee DM, Mahmood U, Weissleder R, Mathis D, Benoist C. Particularities of the vasculature can promote the organ specificity of autoimmune attack. *Nat Immunol* 2006; **7**: 284-292 [PMID: 16444258 DOI: 10.1038/ni1306]

39 **Zhang H**, Xia J, Wang K, Zhang J. Serum autoantibodies in the early detection of esophageal cancer: A systematic review. *Tumour Biol* 2015; **36**: 95-109 [PMID: 25433500 DOI: 10.1007/s13277-014-2878-9]

40 **Fujita Y**, Nakanishi T, Miyamoto Y, Hiramatsu M, Mabuchi H, Miyamoto A, Shimizu A, Takubo T, Tanigawa N. Proteomics-based identification of autoantibody against heat shock protein 70 as a diagnostic marker in esophageal squamous cell carcinoma. *Cancer Lett* 2008; **263**: 280-290 [PMID: 18334280 DOI: 10.1016/j.canlet.2008.01.013]

41 **Zhou SL**, Ku JW, Fan ZM, Yue WB, Du F, Zhou YF, Liu YL, Li Y, Tang S, Hu YL, Hu XP, Hou ZC, Liu J, Liu Y, Feng XS, Wang LD. Detection of autoantibodies to a panel of tumor-associated antigens for the diagnosis values of gastric cardia adenocarcinoma. *Dis Esophagus* 2015; **28**: 371-379 [PMID: 24612004 DOI: 10.1111/dote.12206]

42 **Xu YW**, Chen H, Guo HP, Yang SH, Luo YH, Liu CT, Huang XY, Tang XM, Hong CQ, Li EM, Xu LY, Peng YH. Combined detection of serum autoantibodies as diagnostic biomarkers in esophagogastric junction adenocarcinoma. *Gastric Cancer* 2019; **22**: 546-557 [PMID: 30426295 DOI: 10.1007/s10120-018-0894-y]

43 **Xu YW**, Peng YH, Chen B, Wu ZY, Wu JY, Shen JH, Zheng CP, Wang SH, Guo HP, Li EM, Xu LY. Autoantibodies as potential biomarkers for the early detection of esophageal squamous cell carcinoma. *Am J Gastroenterol* 2014; **109**: 36-45 [PMID: 24296751 DOI: 10.1038/ajg.2013.384]

44 **Zhou SL**, Yue WB, Fan ZM, Du F, Liu BC, Li B, Han XN, Ku JW, Zhao XK, Zhang P, Cui J, Zhou FY, Zhang LQ, Fan XP, Zhou YF, Zhu LL, Liu HY, Wang LD. Autoantibody detection to tumor-associated antigens of P53, IMP1, P16, cyclin B1, P62, C-myc, Survivn, and Koc for the screening of high-risk subjects and early detection of esophageal squamous cell carcinoma. *Dis Esophagus* 2014; **27**: 790-797 [PMID: 24147952 DOI: 10.1111/dote.12145]

45 **Sahin U**, Türeci O, Schmitt H, Cochlovius B, Johannes T, Schmits R, Stenner F, Luo G, Schobert I, Pfreundschuh M. Human neoplasms elicit multiple specific immune responses in the autologous host. *Proc Natl Acad Sci U S A* 1995; **92**: 11810-11813 [PMID: 8524854 DOI: 10.1073/pnas.92.25.11810]

46 **Klade CS**, Voss T, Krystek E, Ahorn H, Zatloukal K, Pummer K, Adolf GR. Identification of tumor antigens in renal cell carcinoma by serological proteome analysis. *Proteomics* 2001; **1**: 890-898 [PMID: 11503213 DOI: 10.1002/1615-9861(200107)1:7<890::aid-prot890>3.0.co;2-z]

47 **Mintz PJ**, Kim J, Do KA, Wang X, Zinner RG, Cristofanilli M, Arap MA, Hong WK, Troncoso P, Logothetis CJ, Pasqualini R, Arap W. Fingerprinting the circulating repertoire of antibodies from cancer patients. *Nat Biotechnol* 2003; **21**: 57-63 [PMID: 12496764 DOI: 10.1038/nbt774]

48 **Kijanka G**, Murphy D. Protein arrays as tools for serum autoantibody marker discovery in cancer. *J Proteomics* 2009; **72**: 936-944 [PMID: 19258055 DOI: 10.1016/j.jprot.2009.02.006]

49 **Hardouin J**, Lasserre JP, Sylvius L, Joubert-Caron R, Caron M. Cancer immunomics: From serological proteome analysis to multiple affinity protein profiling. *Ann N Y Acad Sci* 2007; **1107**: 223-230 [PMID: 17804550 DOI: 10.1196/annals.1381.024]

50 **Shimada H**, Nakashima K, Ochiai T, Nabeya Y, Takiguchi M, Nomura F, Hiwasa T. Serological identification of tumor antigens of esophageal squamous cell carcinoma. *Int J Oncol* 2005; **26**: 77-86 [PMID: 15586227 DOI: 10.3892/ijo.26.1.77]

51 **Gao H**, Zheng Z, Mao Y, Wang W, Qiao Y, Zhou L, Liu F, He H, Zhao X. Identification of tumor antigens that elicit a humoral immune response in the sera of Chinese esophageal squamous cell carcinoma patients by modified serological proteome analysis. *Cancer Lett* 2014; **344**: 54-61 [PMID: 24157810 DOI: 10.1016/j.canlet.2013.10.007]

52 **Macdonald IK**, Parsy-Kowalska CB, Chapman CJ. Autoantibodies: Opportunities for Early Cancer Detection. *Trends Cancer* 2017; **3**: 198-213 [PMID: 28718432 DOI: 10.1016/j.trecan.2017.02.003]

53 **Qiu J**, Keyser B, Lin ZT, Wu T. Autoantibodies as Potential Biomarkers in Breast Cancer. *Biosensors (Basel)* 2018; **8**: pii: E67 [PMID: 30011807 DOI: 10.3390/bios8030067]

54 **Hong Y**, Huang J. Autoantibodies against tumor-associated antigens for detection of hepatocellular carcinoma. *World J Hepatol* 2015; **7**: 1581-1585 [PMID: 26085917 DOI: 10.4254/wjh.v7.i11.1581]

55 **Zhang HF**, Qin JJ, Ren PF, Shi JX, Xia JF, Ye H, Wang P, Song CH, Wang KJ, Zhang JY. A panel of autoantibodies against multiple tumor-associated antigens in the immunodiagnosis of esophageal squamous cell cancer. *Cancer Immunol Immunother* 2016; **65**: 1233-1242 [PMID: 27553002 DOI: 10.1007/s00262-016-1886-6]

56 **Mischak H**, Allmaier G, Apweiler R, Attwood T, Baumann M, Benigni A, Bennett SE, Bischoff R, Bongcam-Rudloff E, Capasso G, Coon JJ, D'Haese P, Dominiczak AF, Dakna M, Dihazi H, Ehrich JH, Fernandez-Llama P, Fliser D, Frokiaer J, Garin J, Girolami M, Hancock WS, Haubitz M, Hochstrasser D, Holman RR, Ioannidis JP, Jankowski J, Julian BA, Klein JB, Kolch W, Luider T, Massy Z, Mattes WB, Molina F, Monsarrat B, Novak J, Peter K, Rossing P, Sánchez-Carbayo M, Schanstra JP, Semmes OJ, Spasovski G, Theodorescu D, Thongboonkerd V, Vanholder R, Veenstra TD, Weissinger E, Yamamoto T, Vlahou A. Recommendations for biomarker identification and qualification in clinical proteomics. *Sci Transl Med* 2010; **2**: 46ps42 [PMID: 20739680 DOI: 10.1126/scitranslmed.3001249]

57 **Xiu Y**, Sun B, Jiang Y, Wang A, Liu L, Liu Y, Sun S, Huangfu M. Diagnostic Value of the Survivin Autoantibody in Four Types of Malignancies. *Genet Test Mol Biomarkers* 2018; **22**: 384-389 [PMID: 29924656 DOI: 10.1089/gtmb.2017.0278]

58 **Qin JJ**, Wang XR, Wang P, Ren PF, Shi JX, Zhang HF, Xia JF, Wang KJ, Song CH, Dai LP, Zhang JY. Mini-array of multiple tumor-associated antigens (TAAs) in the immunodiagnosis of esophageal cancer. *Asian Pac J Cancer Prev* 2014; **15**: 2635-2640 [PMID: 24761876 DOI: 10.7314/APJCP.2014.15.6.2635]

59 **Megliorino R**, Shi FD, Peng XX, Wang X, Chan EK, Tan EM, Zhang JY. Autoimmune response to anti-apoptotic protein survivin and its association with antibodies to p53 and c-myc in cancer detection. *Cancer Detect Prev* 2005; **29**: 241-248 [PMID: 15896923 DOI: 10.1016/j.cdp.2005.03.002]

60 **Zhang JB**, Cao M, Chen J, Ye SR, Xie K, He X, Ma XL, Zhang J, Yie SM. Serum anti-TOPO48 autoantibody as a biomarker for early diagnosis and prognosis in patients with esophageal squamous cell carcinoma. *Clin Res Hepatol Gastroenterol* 2018; **42**: 276-284 [PMID: 29170084 DOI:

10.1016/j.clinre.2017.09.007]
61 **Xu YW**, Peng YH, Ran LQ, Zhai TT, Guo HP, Qiu SQ, Chen HL, Wu ZY, Li EM, Xie JJ. Circulating levels of autoantibodies against L1-cell adhesion molecule as a potential diagnostic biomarker in esophageal squamous cell carcinoma. *Clin Transl Oncol* 2017; **19**: 898-906 [PMID: 28181176 DOI: 10.1007/s12094-017-1623-4]
62 **Li L**, Liu M, Lin JB, Hong XB, Chen WX, Guo H, Xu LY, Xu YW, Li EM, Peng YH. Diagnostic Value of Autoantibodies against Ezrin in Esophageal Squamous Cell Carcinoma. *Dis Markers* 2017; **2017**: 2534648 [PMID: 28298808 DOI: 10.1155/2017/2534648]
63 **Xu YW**, Liu CT, Huang XY, Huang LS, Luo YH, Hong CQ, Guo HP, Xu LY, Peng YH, Li EM. Serum Autoantibodies against STIP1 as a Potential Biomarker in the Diagnosis of Esophageal Squamous Cell Carcinoma. *Dis Markers* 2017; **2017**: 5384091 [PMID: 28852266 DOI: 10.1155/2017/5384091]
64 **Chen WX**, Hong XB, Hong CQ, Liu M, Li L, Huang LS, Xu LY, Xu YW, Peng YH, Li EM. Tumor-associated autoantibodies against Fascin as a novel diagnostic biomarker for esophageal squamous cell carcinoma. *Clin Res Hepatol Gastroenterol* 2017; **41**: 327-332 [PMID: 27956255 DOI: 10.1016/j.clinre.2016.10.011]
65 **Peng YH**, Xu YW, Guo H, Huang LS, Tan HZ, Hong CQ, Li SS, Xu LY, Li EM. Combined detection of serum Dickkopf-1 and its autoantibodies to diagnose esophageal squamous cell carcinoma. *Cancer Med* 2016; **5**: 1388-1396 [PMID: 26988995 DOI: 10.1002/cam4.702]
66 **Jin Y**, Guan S, Liu L, Sun S, Lee KH, Wei J. Anti-p16 autoantibodies may be a useful biomarker for early diagnosis of esophageal cancer. *Asia Pac J Clin Oncol* 2015; **11**: e37-e41 [PMID: 24811068 DOI: 10.1111/ajco.12198]
67 **Looi K**, Megliorino R, Shi FD, Peng XX, Chen Y, Zhang JY. Humoral immune response to p16, a cyclin-dependent kinase inhibitor in human malignancies. *Oncol Rep* 2006; **16**: 1105-1110 [PMID: 17016600 DOI: 10.3892/or.16.5.1105]
68 **Chai Y**, Peng B, Dai L, Qian W, Zhang Y, Zhang JY. Autoantibodies response to MDM2 and p53 in the immunodiagnosis of esophageal squamous cell carcinoma. *Scand J Immunol* 2014; **80**: 362-368 [PMID: 24965442 DOI: 10.1111/sji.12202]
69 **Cai HY**, Wang XH, Tian Y, Gao LY, Zhang LJ, Zhang ZY. Changes of serum p53 antibodies and clinical significance of radiotherapy for esophageal squamous cell carcinoma. *World J Gastroenterol* 2008; **14**: 4082-4086 [PMID: 18609695 DOI: 10.3748/wjg.14.4082]
70 **Müller M**, Meyer M, Schilling T, Ulsperger E, Lehnert T, Zentgraf H, Stremmel W, Volkmann M, Galle PR. Testing for anti-p53 antibodies increases the diagnostic sensitivity of conventional tumor markers. *Int J Oncol* 2006; **29**: 973-980 [PMID: 16964393 DOI: 10.3892/ijo.29.4.973]
71 **Shimada H**, Ochiai T, Nomura F; Japan p53 Antibody Research Group. Titration of serum p53 antibodies in 1,085 patients with various types of malignant tumors: A multiinstitutional analysis by the Japan p53 Antibody Research Group. *Cancer* 2003; **97**: 682-689 [PMID: 12548611 DOI: 10.1002/cncr.11092]
72 **Shimada H**, Nabeya Y, Okazumi S, Matsubara H, Funami Y, Shiratori T, Hayashi H, Takeda A, Ochiai T. Prognostic significance of serum p53 antibody in patients with esophageal squamous cell carcinoma. *Surgery* 2002; **132**: 41-47 [PMID: 12110794 DOI: 10.1067/msy.2002.125307]
73 **Ralhan R**, Arora S, Chattopadhyay TK, Shukla NK, Mathur M. Circulating p53 antibodies, p53 gene mutational profile and product accumulation in esophageal squamous-cell carcinoma in India. *Int J Cancer* 2000; **85**: 791-795 [PMID: 10709097 DOI: 10.1002/(sici)1097-0215(20000315)85:6<791::aid-ijc9>3.0.co;2-k]
74 **Shimada H**, Takeda A, Arima M, Okazumi S, Matsubara H, Nabeya Y, Funami Y, Hayashi H, Gunji Y, Suzuki T, Kobayashi S, Ochiai T. Serum p53 antibody is a useful tumor marker in superficial esophageal squamous cell carcinoma. *Cancer* 2000; **89**: 1677-1683 [PMID: 11042560 DOI: 10.1002/1097-0142(20001015)89:8<1677::AID-CNCR5>3.0.CO;2-9]
75 **Hagiwara N**, Onda M, Miyashita M, Sasajima K. Detection of circulating anti-p53 antibodies in esophageal cancer patients. *J Nippon Med Sch* 2000; **67**: 110-117 [PMID: 10754600 DOI: 10.1272/jnms.67.110]
76 **Shimada H**, Nakajima K, Ochiai T, Koide Y, Okazumi SI, Matsubara H, Takeda A, Miyazawa Y, Arima M, Isono K. Detection of serum p53 antibodies in patients with esophageal squamous cell carcinoma: Correlation with clinicopathologic features and tumor markers. *Oncol Rep* 1998; **5**: 871-874 [PMID: 9625835 DOI: 10.3892/or.5.4.871]
77 **Sobti RC**, Parashar K. A study on p53 protein and anti-p53 antibodies in the sera of patients with oesophageal cancer. *Mutat Res* 1998; **422**: 271-277 [PMID: 9838161 DOI: 10.1016/s0027-5107(98)00207-3]
78 **Cawley HM**, Meltzer SJ, De Benedetti VM, Hollstein MC, Muehlbauer KR, Liang L, Bennett WP, Souza RF, Greenwald BD, Cottrell J, Salabes A, Bartsch H, Trivers GE. Anti-p53 antibodies in patients with Barrett's esophagus or esophageal carcinoma can predate cancer diagnosis. *Gastroenterology* 1998; **115**: 19-27 [PMID: 9649454 DOI: 10.1016/s0016-5085(98)70360-9]
79 **Oshima Y**, Shimada H, Yajima S, Nanami T, Matsushita K, Nomura F, Kainuma O, Takiguchi N, Soda H, Ueda T, Iizasa T, Yamamoto N, Yamamoto H, Nagata M, Yokoi S, Tagawa M, Ohtsuka S, Kuwajima A, Murakami A, Kaneko H. NY-ESO-1 autoantibody as a tumor-specific biomarker for esophageal cancer: Screening in 1969 patients with various cancers. *J Gastroenterol* 2016; **51**: 30-34 [PMID: 25906289 DOI: 10.1007/s00535-015-1078-8]
80 **Fujita S**, Wada H, Jungbluth AA, Sato S, Nakata T, Noguchi Y, Doki Y, Yasui M, Sugita Y, Yasuda T, Yano M, Ono T, Chen YT, Higashiyama M, Gnjatic S, Old LJ, Nakayama E, Monden M. NY-ESO-1 expression and immunogenicity in esophageal cancer. *Clin Cancer Res* 2004; **10**: 6551-6558 [PMID: 15475443 DOI: 10.1158/1078-0432.ccr-04-0819]
81 **Zhou JH**, Zhang B, Kernstine KH, Zhong L. Autoantibodies against MMP-7 as a novel diagnostic biomarker in esophageal squamous cell carcinoma. *World J Gastroenterol* 2011; **17**: 1373-1378 [PMID: 21455340 DOI: 10.3748/wjg.v17.i10.1373]
82 **Zhang J**, Wang K, Zhang J, Liu SS, Dai L, Zhang JY. Using proteomic approach to identify tumor-associated proteins as biomarkers in human esophageal squamous cell carcinoma. *J Proteome Res* 2011; **10**: 2863-2872 [PMID: 21517111 DOI: 10.1021/pr200141c]
83 **Fujita Y**, Nakanishi T, Hiramatsu M, Mabuchi H, Miyamoto Y, Miyamoto A, Shimizu A, Tanigawa N. Proteomics-based approach identifying autoantibody against peroxiredoxin VI as a novel serum marker in esophageal squamous cell carcinoma. *Clin Cancer Res* 2006; **12**: 6415-6420 [PMID: 17085654 DOI: 10.1158/1078-0432.ccr-06-1315]
84 **Liu WL**, Guo XZ, Zhang LJ, Wang JY, Zhang G, Guan S, Chen YM, Kong QL, Xu LH, Li MZ, Song LB,

Zeng MS. Prognostic relevance of Bmi-1 expression and autoantibodies in esophageal squamous cell carcinoma. *BMC Cancer* 2010; **10**: 467 [PMID: 20809956 DOI: 10.1186/1471-2407-10-467]
85 **Li Y**, Zhang Q, Peng B, Shao Q, Qian W, Zhang JY. Identification of glutathione S-transferase omega 1 (GSTO1) protein as a novel tumor-associated antigen and its autoantibody in human esophageal squamous cell carcinoma. *Tumour Biol* 2014; **35**: 10871-10877 [PMID: 25085586 DOI: 10.1007/s13277-014-2394-y]
86 **Ren P**, Ye H, Dai L, Liu M, Liu X, Chai Y, Shao Q, Li Y, Lei N, Peng B, Yao W, Zhang J. Peroxiredoxin 1 is a tumor-associated antigen in esophageal squamous cell carcinoma. *Oncol Rep* 2013; **30**: 2297-2303 [PMID: 24009050 DOI: 10.3892/or.2013.2714]
87 **Ye L**, Guan S, Zhang C, Lee KH, Sun S, Wei J, Liu B. Circulating autoantibody to FOXP3 may be a potential biomarker for esophageal squamous cell carcinoma. *Tumour Biol* 2013; **34**: 1873-1877 [PMID: 23483489 DOI: 10.1007/s13277-013-0729-8]
88 **Guan S**, Liu B, Zhang C, Lee KH, Sun S, Wei J. Circulating autoantibody to CD25 may be a potential biomarker for early diagnosis of esophageal squamous cell carcinoma. *Clin Transl Oncol* 2013; **15**: 825-829 [PMID: 23423807 DOI: 10.1007/s12094-013-1007-3]
89 **Cheng Y**, Xu J, Guo J, Jin Y, Wang X, Zhang Q, Liu L. Circulating autoantibody to ABCC3 may be a potential biomarker for esophageal squamous cell carcinoma. *Clin Transl Oncol* 2013; **15**: 398-402 [PMID: 23054755 DOI: 10.1007/s12094-012-0941-9]
90 **Zhang B**, Zhang Z, Zhang X, Gao X, Kernstine KH, Zhong L. Serological antibodies against LY6K as a diagnostic biomarker in esophageal squamous cell carcinoma. *Biomarkers* 2012; **17**: 372-378 [PMID: 22515502 DOI: 10.3109/1354750X.2012.680609]
91 **Kagaya A**, Shimada H, Shiratori T, Kuboshima M, Nakashima-Fujita K, Yasuraoka M, Nishimori T, Kurei S, Hachiya T, Murakami A, Tamura Y, Nomura F, Ochiai T, Matsubara H, Takiguchi M, Hiwasa T. Identification of a novel SEREX antigen family, ECSA, in esophageal squamous cell carcinoma. *Proteome Sci* 2011; **9**: 31 [PMID: 21696638 DOI: 10.1186/1477-5956-9-31]
92 **Dong J**, Zeng BH, Xu LH, Wang JY, Li MZ, Zeng MS, Liu WL. Anti-CDC25B autoantibody predicts poor prognosis in patients with advanced esophageal squamous cell carcinoma. *J Transl Med* 2010; **8**: 81 [PMID: 20813067 DOI: 10.1186/1479-5876-8-81]
93 **Liu WL**, Zhang G, Wang JY, Cao JY, Guo XZ, Xu LH, Li MZ, Song LB, Huang WL, Zeng MS. Proteomics-based identification of autoantibody against CDC25B as a novel serum marker in esophageal squamous cell carcinoma. *Biochem Biophys Res Commun* 2008; **375**: 440-445 [PMID: 18722351 DOI: 10.1016/j.bbrc.2008.08.039]
94 **Tsunemi S**, Nakanishi T, Fujita Y, Bouras G, Miyamoto Y, Miyamoto A, Nomura E, Takubo T, Tanigawa N. Proteomics-based identification of a tumor-associated antigen and its corresponding autoantibody in gastric cancer. *Oncol Rep* 2010; **23**: 949-956 [PMID: 20204278 DOI: 10.3892/or_00000719]
95 **Shimada H**, Shiratori T, Yasuraoka M, Kagaya A, Kuboshima M, Nomura F, Takiguchi M, Ochiai T, Matsubara H, Hiwasa T. Identification of Makorin 1 as a novel SEREX antigen of esophageal squamous cell carcinoma. *BMC Cancer* 2009; **9**: 232 [PMID: 19604354 DOI: 10.1186/1471-2407-9-232]
96 **Shimada H**, Kagaya A, Shiratori T, Nomura F, Takiguchi M, Matsubara H, Hiwasa T. Detection of anti-CUEC-23 antibodies in serum of patients with esophageal squamous cell carcinoma: A possible new serum marker for esophageal cancer. *J Gastroenterol* 2009; **44**: 691-696 [PMID: 19407926 DOI: 10.1007/s00535-009-0060-8]
97 **Shimada H**, Kuboshima M, Shiratori T, Nabeya Y, Takeuchi A, Takagi H, Nomura F, Takiguchi M, Ochiai T, Hiwasa T. Serum anti-myomegalin antibodies in patients with esophageal squamous cell carcinoma. *Int J Oncol* 2007; **30**: 97-103 [PMID: 17143517 DOI: 10.3892/ijo.30.1.97]
98 **Kuboshima M**, Shimada H, Liu TL, Nomura F, Takiguchi M, Hiwasa T, Ochiai T. Presence of serum tripartite motif-containing 21 antibodies in patients with esophageal squamous cell carcinoma. *Cancer Sci* 2006; **97**: 380-386 [PMID: 16630135 DOI: 10.1111/j.1349-7006.2006.00192.x]
99 **Kuboshima M**, Shimada H, Liu TL, Nakashima K, Nomura F, Takiguchi M, Hiwasa T, Ochiai T. Identification of a novel SEREX antigen, SLC2A1/GLUT1, in esophageal squamous cell carcinoma. *Int J Oncol* 2006; **28**: 463-468 [PMID: 16391802 DOI: 10.3892/ijo.28.2.463]
100 **Nakashima K**, Shimada H, Ochiai T, Kuboshima M, Kuroiwa N, Okazumi S, Matsubara H, Nomura F, Takiguchi M, Hiwasa T. Serological identification of TROP2 by recombinant cDNA expression cloning using sera of patients with esophageal squamous cell carcinoma. *Int J Cancer* 2004; **112**: 1029-1035 [PMID: 15386348 DOI: 10.1002/ijc.20517]
101 **Werner S**, Chen H, Butt J, Michel A, Knebel P, Holleczek B, Zörnig I, Eichmüller SB, Jäger D, Pawlita M, Waterboer T, Brenner H. Evaluation of the diagnostic value of 64 simultaneously measured autoantibodies for early detection of gastric cancer. *Sci Rep* 2016; **6**: 25467 [PMID: 27140836 DOI: 10.1038/srep25467]

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MINIREVIEWS

Hepatic senescence, the good and the bad

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Abstract

Gradual alterations of cell's physiology and functions due to age or exposure to various stresses lead to the conversion of normal cells to senescent cells. Once becoming senescent, the cell stops dividing permanently but remains metabolically active. Cellular senescence does not have a single marker but is characterized mainly by a combination of multiple markers, such as, morphological changes, expression of cell cycle inhibitors, senescence associated β-galactosidase activity, and changes in nuclear membrane. When cells in an organ become senescent, the entire organism can be affected. This may occur through the senescence-associated secretory phenotype (SASP). SASP may exert beneficial or harmful effects on the microenvironment of tissues. Research on senescence has become a very exciting field in cell biology since the link between age-related diseases, including cancer, and senescence has been established. The loss of regenerative and homeostatic capacity of the liver over the age is somehow connected to cellular senescence. The major contributors of senescence properties in the liver are hepatocytes and cholangiocytes. Senescent cells in the liver have been implicated in the etiology of chronic liver diseases including cirrhosis and hepatocellular carcinoma and in the interference of liver regeneration. This review summarizes recently reported findings in the understanding of the molecular mechanisms of senescence and its relationship with liver diseases.

Key words: Senescence; Senescence associated secretory phenotype; Hepatocyte; Cholangiocyte; Hepatic stellate cell; Cell cycle arrest; DNA damage

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Core tip: The liver is a vital organ for metabolic regulation and plays a major role in the nutrient utilization of the whole body. Although scientists have paid much attention to hepatic senescence, the precise mechanisms in, and the contribution of cellular senescence to liver diseases have yet to be clearly defined. In this review, we have

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summarized the mechanism of senescence in general and in the liver. We have also discussed whether hepatocellular senescence is beneficial or harmful to the liver in the light of published findings.

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INTRODUCTION

Definition of senescence

In response to endogenous and exogenous stress, cells in various tissues may enter into a permanent cell cycle arrest. These cells cannot proliferate but remain metabolically active for an extended period of time and are thus termed as senescent cells. Conversion of normal cells into senescent cells occurs throughout the life-time of an organism and plays important roles in tissue homeostasis[1]. In 1961, Leonard Hayflick observed that a human fibroblast cell ceased replication after forty to sixty divisions. As a result of Hayflick's experiments the limited capacity for cellular division in cell culture was termed as the "Hayflick Limit". Later it was found that the cause of the Hayflick Limit was the shortening of telomeres, portions of DNA at the ends of chromosomes, which slowly erode as cells replicate.

Hayflick and Moorhead first introduced the term senescence to describe the limited proliferation ability of human fibroblasts in cell culture. However, cells with senescent properties have been discovered *in vivo* in many types of tissues and the number of senescent cells increases with age[1-6]. Initially senescence was thought to be an artifact in tissue culture with no relevance to the physiology and pathology of an organism. But subsequent studies have proved the importance of senescence in the biological processes, such as embryonic development, tissue repair, tumor suppression and aging[7]. Senescence is now considered as a multistep, dynamic cellular process. When cells are stimulated by senescence-inducing signals, such as oncogene activation, DNA damage, or other stress-mediated signals, they undergo cell cycle arrest or senescence initiation. In the next step, cells with cell cycle arrest undergo chromatin remodeling, present senescence-associated secretory phenotype (SASP), change morphology and gain other characteristics of a full-fledged senescence phenotype. Senescent cells can persist for months[8]. When senescence occurs due to oncogene activation, cell cycle arrest first happens in an autocrine manner, but SASP factors can induce paracrine senescence in other cells at a late stage after the immune system is activated[9,10], thus spreading senescence to neighboring cells across an organ.

There are two hypotheses to explain whether cellular senescence is beneficial or detrimental to an organism. We can consider senescence as a tumor suppressive or anti-cancer process as the senescent cells cannot divide. Therefore, senescence may be good for an organism. On the other hand, cellular senescence may cause loss of regenerative capability of an organ such as the liver. In this respect, it is considered a deleterious process for an organism as it may affect the function and tissue renewal[11]. In either case, more *in vivo* studies on the mechanisms are needed.

Characteristics of senescence

The senescence phenotype is very stable, unresponsive to mitogenic stimuli and resistant to apoptosis[11]. The telomere dysfunction in normal cells can lead to the conversion to replicative senescent cells. Important stimuli like oncogene over-activation, global DNA damage, and oxidative stress may act individually or synergistically to trigger senescence in normal cells[12]. When cells undergo senescence, they are characterized by alterations in morphology, lysosomal activity and gene-expression. These include expression of cell cycle inhibitors, such as $p15^{INK4B}$, $p16^{INK4A}$, and $p21^{Cip1}$, activation of DNA damage response, alterations of chromatin structures and induction of SASP. Usually senescent cells exhibit enlarged, flat morphology and are frequently multi-nucleated[13]. For example, the report from Aravinthan *et al*[14] showed that senescent hepatocytes, which overexpress $p21^{Cip1}$, can be characterized with larger nuclei, compared to non-senescent hepatocytes.

Senescent cells express a higher level of lysosomal β–galactosidase gene (*GLB1*) and show an increased β–galactosidase activity at a suboptimal pH of 6.0 (optimal pH

level is 4-4.5), compared to normal cells[3,15]. Of note, as a single cell based assay, the β-galactosidase activity assay is the most commonly used analysis for the senescence property of cells *in vitro or in vivo*[16]. The cumulative effect of various stresses on the small population of affected cells may lead to conversion of a homogenous population of senescent cells[12].

SASP and senescence

Senescent cells can secrete a large number of proinflammatory cytokines, chemokines, growth factors and proteases that may have a variety of effects on neighboring cells. This phenotype of senescent cells is known as senescence-associated secretory phenotype (SASP). Senescent cells are highly secretory and they execute a diverse set of functions mediated by SASP[17]. As senescent cells are viable and actively secreting cells, they have a profound influence on the surrounding cells. SASP affects senescent cells and their microenvironments in both autocrine and paracrine fashions. SASP factors are highly dynamic in expression and the composition changes over the period of senescence. This change in the composition of SASP in senescent cells may determine whether the effect is beneficial or harmful in the respective organs[17]. The mechanisms of the initiation and regulation of SASP and its influence in inflammation and disease processes have not been fully elucidated yet.

SIGNALING PATHWAYS IN SENESCENCE

The reactive oxygen species (ROS) is considered as one of the main activators of senescence cells[18,19]. The imbalanced ROS may cause oxidative stress and DNA damage, leading to the activation of DNA damage response in the affected cells.

Signaling pathways that control cell cycle arrest

The p53 and Rb signaling pathways may become activated in response to extrinsic and intrinsic stressors, including DNA damage. p53 and Rb can affect transcriptionally both upstream regulators and downstream effectors in the senescence pathway[20]. The inactivation of either p53 or Rb prevents or significantly delays senescence[21] (Figure 1). ATM and ATR, as well as their downstream targets, Chk1 and Chk2, become activated during DNA damage, which in turn activate p53. Activated p53 is considered as one of the main players in converting normal cells to senescent cells. The phosphorylated form of p53 is the essential contributor of replicative senescence in human fibroblast[22]. Acetylation of p53 at Lys161/Lys162 is also important for cell cycle arrest and senescence[23]. Once p53 is activated by posttranslational modification, it in turn activates its transcriptional target, $p21^{Cip1}$ (CDKN1A). $p21^{Cip1}$ is the inhibitor of Cyclin E/CDK2 complex and it promotes cell cycle arrest at G1/S phase of cell cycle (Figure 1). Of note, $p21^{Cip1}$ can be activated by both p53-dependent and p53-independent mechanisms[24].

Retinoblastoma protein (Rb) is a tumor suppressor and a well-established cell cycle regulator. Rb is expressed in all tissues and controls cell cycle progression through interactions with the E2F family of transcription factors[20,25]. Other Rb family members, namely, p107 and p130, also play active and important role in driving senescence independently of Rb[26]. $p21^{Cip1}$ has been found to be both a positive and negative regulator of Rb by regulating either Rb phosphorylation or degradation, respectively[27]. $p16^{INK4A}$ (CDKN2A) inactivates Cyclin-dependent kinases, which phosphorylate Rb, and Rb phosphorylation status in turn has an impact on the expression of $p16^{INK4A}$. In normal cells, Cyclin D and Cyclin E bind to CDK2 and CDK4/6, respectively, since $p21^{Cip1}$ and $p16^{INK4A}$ level remain at the basal level. Hyperphosphorylation of Rb permits the E2F factor to participate in the production of replication proteins and hence cell cycle progress. But a higher level of either $p16^{INK4A}$ and/or $p21^{Cip1}$ can keep Rb in the hypophosphorylated state so that E2F remains bound with it. Therefore, cell cycle is arrested, which ultimately lead to cellular senescence (Figure 1).

Another cell cycle inhibitor, CDKN3 is a phosphatase and dephosphorylates Rb thus ensuring that cell cycle progression is blocked. Thus, CDKN3 is considered as an important contributor to cellular senescence[28]. CDKN3 can also interact with Mdm2 and form a complex with p53 and Mdm2. Once the complex is formed, p53 losses its ability to induce $p21^{Cip1}$[29,30].

G1-phase cell cycle arrest can also be induced by transforming growth factor-beta (TGFβ)[31]. TGFβ can keep Rb in hypophosphorylated state, suggesting that it may suppress Rb phosphorylation and thereby interfering with cell cycle progression[32]. $p15^{INK4B}$(CDKN2B), a homologue of $p16^{INK4A}$, is activated by TGFβ, acts on CyclinD-CDK4/CDK6 complex and contributes to cell cycle arrest, and hence to senescence[33] (Figure 1). Since $p15^{INK4B}$ and $p16^{INK4A}$ have a common mode of action, in a tissue

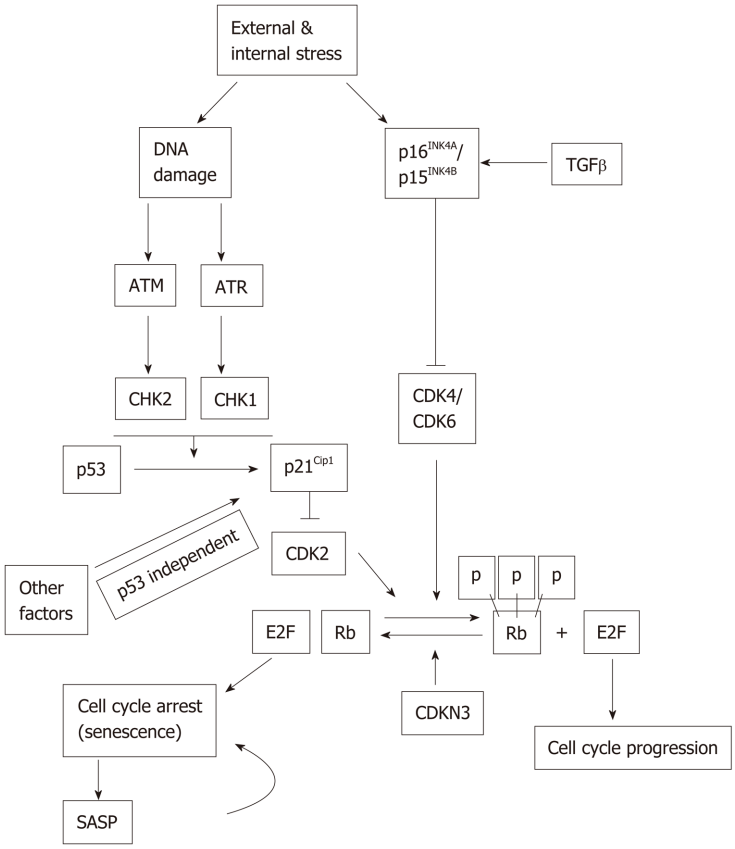

Figure 1 Stress-induced senescence. Both external and internal stresses can induce DNA damage and the activation of $p16^{INK4A}$ and/or $p15^{INK4B}$. DNA damage can activate p53 ataxia telangiectasia mutated (ATM) and ATM and RAD3-related (ATR) pathway. Activated p53 induces $p21^{Cip1}$ expression. Expression of $p21^{Cip1}$ can also be regulated by p53-independent mechanisms. The Cyclin dependent kinases activate Rb but are inhibited by $p15^{INK4B}$/$p16^{INK4A}$ and $p21^{Cip1}$ which leads to cell cycle arrest and senescence. The senescent cells express senescence-associated secretory phenotype (SASP). The SASP factors may induce senescence in neighboring cells in a paracrine fashion. ATM: Ataxia telangiectasia mutated; SASP: Senescence-associated secretory phenotype.

where either one does not express, the other can possibly compensate the functionality.

Signaling pathways that regulate SASP

The connection between senescent cells and the immune system is mediated by SASP. The inflammation signature of SASP include chemokines, cytokines and other immune modulators. SASP is regulated at multiple levels' such as, chromatin modification, transcription, translation, mRNA stability and secretion[34]. The autocrine and paracrine positive feedback loops also regulate the signaling of SASP. Most senescent cells express multiple cytokines such as IL-8, CCL2 (MCP-1), CCL7 (MCP-3), CCL8 (MCP-2), CCL13 (MCP-4), CCL3, CCL16, CCL20, CXCL1, CXCL2, CXCL3 *ect*[35,36]. The most characterized SASP components include multiple pro-inflammatory cytokines, such as interleukin-1 (IL-1), and interleukin-6 (IL-6). IL-1 and IL-6 produced in senescent epithelial and fibroblast cells are capable of inducing cellular senescence in adjacent cells. SASP helps spreading senescence phonotype among nonsenescent cells by creating an inflammatory environment in tissues. The knock-down of IL-6 and IL-8 receptors IL-6R and CXCR2, respectively, prevents senescence[34].

SASP is induced and regulated by several signaling pathways leading to the activation of the nuclear factor-κB (NF-κB) and/or CCAAT/enhancer-binding protein-β (C/EBPβ). In senescent cells, activated NF-κB and C/EBPβ regulate the expression of SASP factors by controlling, mainly at the transcription level, the inflammatory SASP molecules, IL-1α, IL-6 and IL-8. These inflammatory SASP factors can enhance SASP signaling through activating NF-κB , and C/EBPβ in an autocrine feed-forward fashion[37,38] (Figure 2).

NOTCH signaling pathway: This pathway has been implicated as an important regulator of SASP. Works from Hoare *et al*[39] suggested a global upregulation of

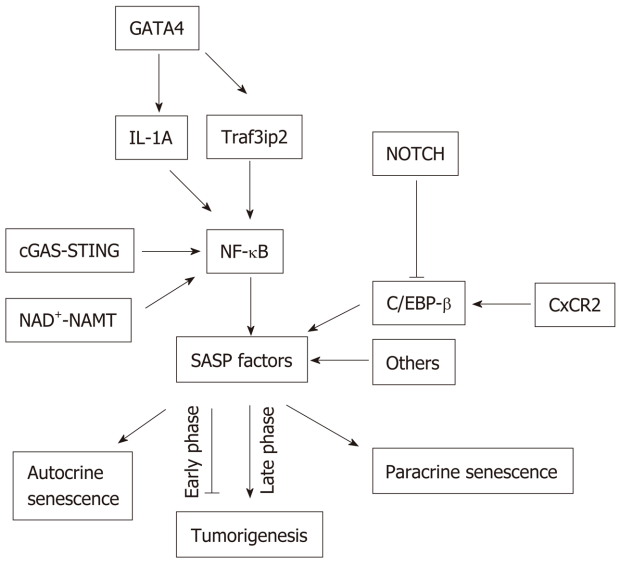

Figure 2 Senescence-associated secretory phenotype signaling pathways. Nuclear factor kappa light chain enhancer of activated B cells can be activated *via* multiple signaling pathways such as the GATA binding protein 4, cyclic GMP-AMP synthase-stimulator of interferon genes, and nicotinamide adenine dinucleotide -nicotinamide phosphoribosyltransferase NAD^+-NAMT pathways, which lead to the expression of senescence-associated secretory phenotype (SASP) proteins. SASP can be positively regulated through C-X-C motif chemokine receptor 2, or negatively by NOTCH *via* CCAAT-enhancer-binding proteins. SASP can induce senescence in both autocrine and paracrine manners. SASP can be anti-tumorigenic in the early phase of senescence, but can be pro-tumorigenic in the late phase of senescence. NF-κB: Nuclear factor kappa light chain enhancer of activated B cells; GATA4: GATA binding protein 4; cGAS-STING: Cyclic GMP-AMP synthase-stimulator of interferon genes; SASP: Senescence-associated secretory phenotype; CXCR2: C-X-C motif chemokine receptor 2; C/EBP: CCAAT-enhancer-binding protein.

NOTCH1 accompanied by dynamic alterations of its downstream activity in senescence. They also proposed NOTCH1 as a master regulator of SASP composition *via* a temporal and functional switch between two different secretomes, TGFβ and pro-inflammatory cytokines through down-regulation of C/EBPβ.

The NOTCH1 was shown to be upregulated in cells undergoing oncogene-induced senescence (OIS), which caused upregulation of TGFβ in the first phase of senescence. The inhibition of NOTCH signaling substantially reduced TGFβ induction in OIS cells. The inhibition of TGFβ in NOTCH1-driven senescent cells prevented the upregulation of the TGFβ-targeted cell cycle inhibitor, $p15^{INK4B}$. In addition, Hoare *et al*[39] found that NOTCH1 negatively regulated C/EBPβ but had no effect on NF-κB pathway in the later phase of senescence. The overexpression of activated Notch1 receptors (N1ICD - NOTCH1 intracellular domain) inhibited the ability of C/EBPβ to induce IL-1, IL-6 and IL-8[39]. Moreover, Hoare *et al*[39] found that inhibiting Notch signaling accelerated clearance of senescent cells in the liver. Therefore, NOTCH signaling pathway may be associated with a complicated mechanism of SASP regulation. But the key questions that remain to be addressed are: (1) Which factor(s) involved in the switching from NOTCH1-TGFβ to NOTCH1-C/EBPβ; (2) What the cross-talk is between NOTCH1-mediated C/EBPβ and NF-kB regulation in senescent cells.

cGAS-STING pathway: cGAS (cGMP-AMP synthase) is a cytosolic DNA sensor and it is activated in response to cytosolic DNA. This activated cGAS binds and activates Stimulator of Interferon Gene (STING). During cellular senescence, the organization of chromatin changes and undergoes degeneration. The integrity of the nuclear envelope is disrupted due to the loss of the nuclear lamina protein Lamin B1. Eventually, small chromatin fragments migrate from nucleus to cytoplasm to become cytoplasmic chromatin fragments (CCF) in senescent cells[40]. cGAS can be activated by any double-stranded DNA irrespective of the sequence[41]. Therefore, CCF from the senescent cells can activate cGAS-STING pathway and upregulate SASP expression via inducing type-1 interferons (IFN-1). The cGAS-STING signaling pathway drives the production of inflammatory SASP cytokines through type I interferon *via* IRF3 and proinflammatory responses *via* NF-κB[40,42], thereby facilitating senescence. Gluck *et al*[43] demonstrated that cGAS deficiency in the liver abrogated the induction of

senescence in hepatocytes, which suggests the importance of cGAS in the process of senescence *in vivo*.

Cecco *et al*.[44] showed that in oncogene-induced senescence and stress-induced premature senescence, activation of retrotransposable element, L1, and IFN-1 occur in the late phase. They found three factors that caused L1 activation, RB1, FOXA1 and TREX1. RB1 expression was declined in senescent cells but was enhanced in proliferating cells. Upregulation of FOXA1 and downregulation of RB1 and TREX1 were found to be associated with L1 activation and stabilization. The activation of L1 ensured a strong activation of INF-1 response in senescent cells. In addition, when the cGAS-STING pathway was disrupted, the INF-1 response was inhibited and SASP response such as the induction of CCL2, IL-6, and MMP3 were downregulated in the late phase of senescent cells.

NAD$^+$-NAMT pathway: NAD$^+$ and the rate-limiting enzyme of its synthesis, nicotinamide phosphoribosyltransferase (NAMPT), have a critical role in ageing and cancer[45-47]. Recently, Nacarelli *et al*[47] demonstrated a mechanism of SASP regulation that involves these factors and HMGA proteins HMGA1 and HMGA2[47]. HMGA proteins are known to regulate the chromatin structure and to promote senescence[48]. Now, these proteins are found to regulate *NAMPT* gene expression by binding at the enhancer element during oncogene-induced senescence of fibroblasts[47]. NAMPT upregulates the expression of proinflammatory SASP fators such as IL-1β, IL-6 and IL-8, which are dependent on the enzymatic activity of NAMPT. This proinflammatory activity are regulated by increased NAD$^+$/NADH ratio. Nacarelli *et al*[47] have also demonstrated that increased NAD$^+$/NADH ratio causes suppression of AMPK, which interferes with p53-mediated inhibition of p38 MAPK. p38 MAPK controls the transcriptional activity of NF-κB by regulating acetylation of p65. The NF-κB mediated proinflammatory activity is therefore enhanced.

In addition to HMGA proteins, another high mobility group protein, HMGB1, has been implicated in the senescence and SASP. Davalos *et al*[49] reported the existence of a relationship between HMGB1 and senescence. They found that altered expression of HMGB1 induced senescence in a p53-dependent manner in fibroblasts. Exogenous HMGB1 treatment activated NF-κB activity and IL-6 secretion whereas depletion of HMGB1 attenuated the senescence phenotype. The disruption of normal HMGB1 levels can induce a p53-dependent cell cycle arrest. Interestingly, secreted HMGB1 is essential for optimal secretion of IL-6 and MMP-3, both are important SASP components.

Other pathways: The other known regulators of SASP are bromodomain containg protein 4 (BRD4), lysine methyltransferase MLL1 and G9A[50]. The recruitment of BRD4 to senescence-activated enhancers located adjacent to SASP genes is probably required to induce SASP[51]. But the upstream signaling that regulate these transcriptional activators are not completely known. SASP helps spreading senescence phonotype among nonsenescent cells by creating an inflammatory environment in tissues.

For SASP regulation, mTOR plays an important role as well. The 4E-BP1 is phosphorylated by mTOR. It is a repressor of translation of mRNA. In order to regulate SASP, 4E-BP1 represses IL-1α and MAP kinase-activated protein kinase 2[52]. The degradation of mRNA of proinflammatory SASP factors is associated with a RNA binding protein, zinc finger protein 36L1 (ZFP36L1); and MAP kinase-activated protein kinase 2 inhibits ZFP36L1[34]. By regulating the stability of SASP mRNA, mTOR regulates SASP in senescent cells.

Autophagy and senescence

Autophagy is a highly regulated cellular program responsible for recycling intracellular proteins and damaged/nonfunctional organelles. Autophagy was found to be activated in senescence in human fibroblasts due to the negative feedback of mTOR pathway[53]. A number of genes (*e.g*., Ulk3 and LC3) associated with autophagy are upregulated in senescent cells and inhibition of autophagy essential genes like Atg5 or Atg7 caused interference of senescence phenotype including SASP[53]. Reciprocally, Kang *et al*[54] showed that knockdown of autophagy essential genes such as Atg7, Atg12 or lysosomal associated membrane protein 2 (LAMP2) caused senescence in human fibroblasts. This senescence pathway was found to be ROS and p53-dependent. In contrast, Garcia-Prat *et al*[55] demonstrated that muscle stem cells maintain a reversible quiescence state and do not enter to senescence when the autophagy mechanism is active. This group showed that the muscle stem cells underwent senescence if autophagy was defective and restoration of autophagy prevented senescence in satellite cells. Therefore, autophagy may both induce and prevent senescence, which may be dependent on cell type or the type of experimental

model. However, it is still an open question as to whether autophagy is necessary for senescence to occur or it inhibits the senescence process.

Kang *et al*[56] reported that a transcription factor, GATA4 played an important role in the cellular senescence mechanism. This group suggests that GATA4 is a senescence and SASP regulator. In normal cells, GATA4 binds to an autophagy adaptor, p62, whichis eliminated by the selective autophagy process. This autophagy process is suppressed when senescence is induced. DNA damage activates ATM and ATR kinases, which inhibit p62-mediated GATA4 degradation. The stabilized GATA4 then activates NF-κB inducers TRAF3IP2 and IL-1A. NF-κB, one of the master regulators of senescence, initiates and maintains SASP, and facilitates senescence. The GATA4 activation is found to be independent of p53 and $p16^{INK4A}$ but is dependent on ATM and ATR kinases. This finding suggests that GATA4 may represent a separate branch of DNA damage repair response that induces SASP and hence favors senescence. Kang *et al*[56] has suggested a clarification on the argument whether autophagy induces or inhibits senescence. Selective autophagy prevents cells from undergoing senescence by limiting GATA4 level when senescence-inducing stimuli, such as irradiation are given but later general autophagy lead to cellular senescence[57].

At least, one more member of the GATA family, GATA6, has been implicated in the senescence mechanism. Perlman *et al*[58] reported that GATA6 overexpression could upregulate the expression of $p21^{Cip1}$ so that the proliferation of vascular smooth muscle cells and fibroblasts were inhibited. Zhang *et al*[59] suggested the existence of a direct relationship between autophagy and GATA6. Autophagy decreased accumulation of GATA6 and p62 was found to be a negative regulator of GATA6 accumulation. Zhang *et al*[59] also determined the relationship between an antimalarial drug, dihydroartemisinin (DHA) and GATA6, and their contribution to the hepatic senescence. DHA treatment enhanced the expression of p53, $p21^{Cip1}$ and $p16^{INK4A}$ and induced senescence in hepatic stellate cells (HSCs) in rat livers. GATA6 accumulation further accelerated the expression of p53 and $p16^{INK4A}$ in DHA-treated livers and promoted senescence. The knock-down of GATA6 by siRNA significantly reduced DHA-induced upregulation of p53 and $p16^{INK4A}$, and thereby the senescence.

SENESCENCE IN THE LIVER

The detailed mechanism and biological function of cellular senescence in liver diseases have not been fully elucidated. But meaningful progresses have been made in this field. Senescence can adversely affect liver functions, cellular viability and tissue regeneration under pathological conditions[60]. Senescence in hepatocytes is well documented in different liver diseases such as cirrhosis[61,62]. Senescence in cholangiocytes[60,63,64] is also known to occur in such biliary diseases as primary biliary cholangitis (PBC) and primary sclerosing cholangitis (PSC). Senescence in HSCs has been reported in fibrosis[65].

Mouse models to study liver cell senescence

There are various mouse models developed to study senescence-associated liver diseases. Demaria *et al*[66] developed a mouse model named $p16^{INK4A\text{-}3MR}$ to visualize and to eliminate senescent cells *in vivo*. The promoter of $p16^{INK4A}$ was used in this model to express a fusion protein, composed of luciferase, red fluorescence protein (RFP) and herpes simplex virus 1 thymidine kinase. This viral thymidine kinase converts ganciclovir (GCV) into an apoptosis inducer. GCV treatment induces p53 accumulation and surges cell surface expression of CD95 and tumor necrosis factor receptor. Utilizing the $p16^{INK4A\text{-}3MR}$ mice and imaging, researchers can investigate a vigorous, but transient, upsurge in senescent cells *in vivo*.

$AhCre^{+}Mdm2^{fl/fl}$ mice[9]: Murine Double Minute 2 (MDM2) interferes the transcriptional activity of p53 and guides degradation of p53 through ubiquitination. The disruption of the p53–MDM2 interaction by knocking out MDM2 genetically leads to p53-mediated cell-cycle arrest through inducing the activation of cell cycle inhibitor, p21Cip1[67]. Deletion of Mdm2 causes upregulation of p53 and thus $p21^{Cip1}$. The AhCre system allows expression of Cre recombinase in hepatocytes in response to β-naphthoflavin, which in turn deletes the floxed Mdm2. This $AhCre^{+}Mdm2^{fl/fl}$ mouse model induces $p21^{Cip1}$-dependent hepatocellular senescence conditionally[9]. The rescue of growth arrest may be achieved by crossing the $AhCre^{+}Mdm2^{fl/fl}$ mouse strain to $p21^{Cip1}$-deficient mouse strain.

K19-$Mdm2^{f/f}$tdTomLSL[60]: In this mouse model, Mdm2 can be conditionally deleted in cholangiocytes as the Cre recombinase expression is under the control of Krt19 promoter. This model exhibits features of biliary diseases upon induction of senescence in cholangiocytes. The Cre activity in senescent cholangiocytes can be

monitored following the expression of tdTomato[60].

Mdr2(Abcb4)(-/-) mouse model is useful for the study of chronic biliary liver disease. The *mdr2* gene encodes a membrane protein that transports phosphatidylcholine. *mdr2*-deficient mice are unable to secrete phosphatidylcholine into the bile by hepatocytes which leads to cholestatic liver disease. Researchers have used this model to study senescence in cholangiocytes and fibrosis in the liver[68-70].

Senescence-accelerated mouse prone8 (SAMP8) is widely studied for aging mechanism, but is also useful for the study of liver diseases because of its phenotype of liver dysfunction[71]. The SAMP8 mouse strain exhibits an oxidative stress-induced age-related phenotype and severe mitochondrial dysfunction related liver pathology[72].

Senescence in hepatocytes

The function and the regenerative capacity of the liver deteriorate with age[73]. The accumulation of reactive oxygen species (ROS) during aging could lead to a significant level of oxidative stress. A number of studies have suggested a causative connection between cellular injury and oxidative stress in aged individuals[74,75]. Intano *et al*[76] reported that DNA damage could be caused by ROS and the repair response was slowed down in aged mouse hepatocytes. ROS and DNA damage trigger cells to overexpress cell cycle inhibitors and halt proliferation of the damaged cells by inducing senescence. The liver can repair and regenerate normally if the damage is mild. But when the degree of damage to the liver is severe, hepatocytes lose regenerative capacity, and undergo necrosis, apoptosis or senescence[11,77].

Hepatocellular senescence can cause remarkable changes in tissue homeostasis, and microenvironment through SASP, which may lead to fibrosis and hepatocellular carcinoma. In the early stage of liver diseases, hepatocellular senescence may serve as a protective role by blocking proliferation of affected or injured liver cells. Therefore, the risk for these affected cells to be transformed to cancer cells would be reduced. But at the later stage of the diseases, senescent cells secrete SASP factors with an abnormal level of proinflammatory cytokine/chemokines, such as CCL2, and TGFβ, which can promote the conversion of non-senescent cells to senescent cells in a paracrine manner. Rudolph and DePinho have hypothesized that chronic liver injury causes progressive and repetitive liver damage and regeneration, which leads to accumulation of shorter telomeres in hepatocytes. This in turn may lead to hepatocellular senescence and ultimately cirrhosis[78]. In addition, Wiemann *et al*[62] showed that telomere shortening and hepatocellular senescence were positively correlated with progression of fibrosis in human cirrhosis samples, which denoted hepatic senescence as a general marker of cirrhosis.

The accumulation of fat in hepatocytes can promote telomere shortening and DNA damage, which may be mediated by oxidative stress. The critically shorter telomere and damaged DNA may induce senescence in hepatocytes[11,14]. Aravinthan *et al*[14] demonstrated that hepatocytes in non-alcohol-related fatty liver disease (NAFLD) could enter the senescent state after they overexpressed $p21^{Cip1}$, and presented a larger nuclear area and γ-H2AX positive foci. The presence of hepatocyte senescence positively correlated not only with the fibrotic stage of the liver but also with the diabetic state associated with NAFLD.

Any repetitive wave of insult that can cause damage to hepatocytes such as alcohol intake, hepatitis viral infection, immune disorder or autophagy deficiency, may compromise liver regeneration and promote senescence in hepatocytes and other types of cells[64]. The normal liver contains a basal level of senescent hepatocytes (3%-7%), but in chronic hepatitis and cirrhosis the liver may exhibit 50%-100% of hepatocytes in senescence[61,62]. The generation of fibrotic scars in cirrhosis is thought to be the consequence of hepatocyte senescence, which may be used as a marker for cirrhosis[62].

Teoh *et al*[79] reported the induction of p53, $p21^{Cip1}$ and $p16^{INK4A}$ in senescent hepatocytes during chronic liver diseases but the detailed mechanism was not studied. Recently Bird *et al*[9] published a seminal article showing that acute liver injury could also induce hepatocyte senescence. Using a hepatocyte specific senescence induction model (AhCre-$Mdm2^{fl/fl}$) they found that hepatocytes undergoing cell-autonomous senescence can transfer senescence properties to normal hepatocytes *via* a SASP factor, CCL2, which stimulates the production of TGFβ from hepatic macrophages.

The TGFβ pathway was found to be activated in senescent hepatocytes in human fulminant liver failure. The targets of TGFβ pathway, SMAD7 and pSMAD2/3, were upregulated in hepatocytes near the necrotic area during acute liver injury. The interference of monocyte recruitment with an anti-CCL2 antibody in this model led to reduction of senescence spreading from senescent hepatocytes to normal hepatocytes, and enhanced hepatocyte proliferation. This finding suggests that TGFβ and CCL2

can work together in a non-cell-autonomous senescence pathway. The inhibition of TGFβ-TGFβ-R1 interaction by specific inhibitors or the depletion of macrophages by Liposomal Clodronate reduced senescence progress and enhanced liver regeneration[9]. This study thus suggests that the senescence process in acute liver injury may be therapeutically manageable.

Senescent hepatocytes can recruit and activate immune cells using their SASP property. The activated immune cells can remove senescent hepatocytes, which is termed as ''senescence surveillance'', hence preventing malignant transformation[80,81]. Hepatic senescence harboring additional mutations like, p53 mutation, can cause aggressive hepatocellular carcinoma (HCC)[82]. Restoration of p53 in liver tumors can activate immune cell-mediated clearance of senescent tumor cells[83,84]. Kang *et al*[80] found that $CD4^+$ T cells clear senescent hepatocytes with the help of activated monocytes/ macrophages, which also suggested to the importance of senescence surveillance as an antitumor barrier for the HCC.

Senescence in cholangiocytes

The existence of senescent cholangiocytes and their potential deleterious effects in biliary diseases (PBC and PSC) have been described[85,86]. To have a better understanding of the detailed mechanism of cellular senescence in cholangiopathies, an *in vivo* model (K19-Cre-$Mdm2^{f/f}$)[60] was recently created to study the mechanism of cholangiocyte mediated biliary senescence in the liver by conditionally activating senescence in cholangiocytes. The cell-autonomous senescence, where the induction of $p21^{Cip1}$ and $p16^{INK4A}$ are dependent on p53 induction in cholangiocytes, results in the conversion of normal hepatocytes of close proximity into non-cell-autonomous senescence, where the induction of $p21^{Cip1}$ and $p16^{INK4A}$ are independent on p53[60]. As in the AhCre-$Mdm2^{fl/fl}$ model this paracrine senescence was found to be TGFβ-dependent as the blocking of TGFβ signaling pathway partially interfered with the paracrine senescence effect. The involvement of other SASP factors in addition to TGFβ may need to be explored in additional details.

Both studies by Ferreira-Gonzalez *et al*[60] and Bird *et al*[9] have indicated the importance of TGFβ in the transmission of senescence properties from senescent cells to otherwise normal cells. TGFβ has been shown to be involved in the liver disease progression from injury to hepatocellular carcinoma[87]. Therefore, TGFβ *via* small molecule inhibitors could be a valuable therapeutic target to treat cholangiopathies and hepatocellular injury and preventing senescence-associated tumorigenesis.

In cholestatic liver diseases and cholangiopathies, the bile acid (BA) secretion can be defective and bile acids may not reach to intestine, which may cause accumulation of bile acids in the liver, and cholestatic liver injury, which in turn can induce senescence in liver cells[88,89]. Whether a specific BA species can directly cause senescence is not known. *In vitro* treatment of bile duct epithelial cells with deoxycholic acid (DCA) and lithocholic acid (LCA) could not induce SASP. BA may induce senescence in other cells, such as HSCs.

Senescence in HSC

HSCs are in quiescent state in normal healthy livers but become activated following liver injury. Activated HSC play an important role in liver fibrosis. There are three possible fates for the activated and fibrogenic HSC: (1) Apoptosis, to limit the HSC involvement in extracellular matrix (ECM) deposition; (2) Reversal from the activated status to quiescence; and (3) Senescence, to limit the degree of fibrosis in the liver. Multiple inducers of senescence in HSC have been reported, including IL-22, CCN1 (CYR61), retinoic acid, and Substance P (SP)[69,90].

Hepatocellular fibrosis often results from the excessive deposition of ECM by activated stellate cells. The general concept is that senescent hepatocytes are associated with fibrosis, impaired liver function and increased mortality. On the other hand, Krizhanovsky *et al*[65] reported that in response to liver injury, activated HSC initially proliferated and contributed to ECM deposition. However, senescent HSC could limit the extent of fibrosis. Moreover, they found that if HSCs were deficient in key senescent genes in the p53 or Rb pathways, these cells continued to proliferate and secreted excessive ECM. These finding suggest that senescent HSCs are probably beneficial for the injured liver but the senescent hepatocytes are not.

A secondary bile acid, DCA can induce SASP in HSC[91]. The enterohepatic circulation of DCA seemed important for the senescence of HSC. DCA can induce DNA damage by upregulating ROS, which may lead to senescence in HSC but the underlying mechanism is unclear. It is not known if the bile acids can induce senescence in other liver cells.

When HSC proliferate, they secrete molecules that form ECM such as collagens (*e.g*., Col1A) and matrix metalloproteins (MMPs). In acute liver injury, senescent HSC may have antifibrotic effects by secreting matrix metalloprotenases that digest MMPs

and collagens. However, senescent cells of other origins (non-HSC) can express matrix metalloprotenase inhibitors, which would promote fibrosis by antagonizing the effect of senescent HSC. Therefore, detailed studies are required to understand the balance between fibrogenic and non-fibrogenic SASP in senescent HSC and in senescent non-HSC.

CONCLUSION

An increasing number of research on hepatocellular senescence have indicated the importance and significance of the senescence in the liver. The detailed mechanisms of hepatic senescence are yet to be fully determined. Exploring the regulatory mechanisms of SASP discussed above in the liver will provide an important insight to fight against hepatic inflammation, fibrosis or cancer in the liver tissues. Understanding the molecular mechanisms of cellular senescence in liver diseases will help to develop important tools for better diagnosis and treatment of the liver diseases.

REFERENCES

1 **He S**, Sharpless NE. Senescence in Health and Disease. *Cell* 2017; **169**: 1000-1011 [PMID: 28575665 DOI: 10.1016/j.cell.2017.05.015]
2 **Campisi J**. Aging, cellular senescence, and cancer. *Annu Rev Physiol* 2013; **75**: 685-705 [PMID: 23140366 DOI: 10.1146/annurev-physiol-030212-183653]
3 **Dimri GP**, Lee X, Basile G, Acosta M, Scott G, Roskelley C, Medrano EE, Linskens M, Rubelj I, Pereira-Smith O. A biomarker that identifies senescent human cells in culture and in aging skin in vivo. *Proc Natl Acad Sci USA* 1995; **92**: 9363-9367 [PMID: 7568133]
4 **Krishnamurthy J**, Torrice C, Ramsey MR, Kovalev GI, Al-Regaiey K, Su L, Sharpless NE. Ink4a/Arf expression is a biomarker of aging. *J Clin Invest* 2004; **114**: 1299-1307 [PMID: 15520862 DOI: 10.1172/JCI22475]
5 **Hayflick L**. The limited in vitro lifetime of human diploid cell strains. *Exp Cell Res* 1965; **37**: 614-636 [PMID: 14315085]
6 **Hayflick L**, Moorhead PS. The serial cultivation of human diploid cell strains. *Exp Cell Res* 1961; **25**: 585-621 [PMID: 13905658]
7 **van Deursen JM**. The role of senescent cells in ageing. *Nature* 2014; **509**: 439-446 [PMID: 24848057 DOI: 10.1038/nature13193]
8 **Childs BG**, Li H, van Deursen JM. Senescent cells: a therapeutic target for cardiovascular disease. *J Clin Invest* 2018; **128**: 1217-1228 [PMID: 29608141 DOI: 10.1172/JCI95146]
9 **Bird TG**, Müller M, Boulter L, Vincent DF, Ridgway RA, Lopez-Guadamillas E, Lu WY, Jamieson T, Govaere O, Campbell AD, Ferreira-Gonzalez S, Cole AM, Hay T, Simpson KJ, Clark W, Hedley A, Clarke M, Gentaz P, Nixon C, Bryce S, Kiourtis C, Sprangers J, Nibbs RJB, Van Rooijen N, Bartholin L, McGreal SR, Apte U, Barry ST, Iredale JP, Clarke AR, Serrano M, Roskams TA, Sansom OJ, Forbes SJ. TGFβ inhibition restores a regenerative response in acute liver injury by suppressing paracrine senescence. *Sci Transl Med* 2018; **10** [PMID: 30111642 DOI: 10.1126/scitranslmed.aan1230]
10 **Acosta JC**, Banito A, Wuestefeld T, Georgilis A, Janich P, Morton JP, Athineos D, Kang TW, Lasitschka F, Andrulis M, Pascual G, Morris KJ, Khan S, Jin H, Dharmalingam G, Snijders AP, Carroll T, Capper D, Pritchard C, Inman GJ, Longerich T, Sansom OJ, Benitah SA, Zender L, Gil J. A complex secretory program orchestrated by the inflammasome controls paracrine senescence. *Nat Cell Biol* 2013; **15**: 978-990 [PMID: 23770676 DOI: 10.1038/ncb2784]
11 **Campisi J**, d'Adda di Fagagna F. Cellular senescence: when bad things happen to good cells. *Nat Rev Mol Cell Biol* 2007; **8**: 729-740 [PMID: 17667954 DOI: 10.1038/nrm2233]
12 **Ben-Porath I**, Weinberg RA. The signals and pathways activating cellular senescence. *Int J Biochem Cell Biol* 2005; **37**: 961-976 [PMID: 15743671 DOI: 10.1016/j.biocel.2004.10.013]
13 **Carnero A**. Markers of cellular senescence. *Methods Mol Biol* 2013; **965**: 63-81 [PMID: 23296651 DOI: 10.1007/978-1-62703-239-1_4]
14 **Aravinthan A**, Scarpini C, Tachtatzis P, Verma S, Penrhyn-Lowe S, Harvey R, Davies SE, Allison M, Coleman N, Alexander G. Hepatocyte senescence predicts progression in non-alcohol-related fatty liver disease. *J Hepatol* 2013; **58**: 549-556 [PMID: 23142622 DOI: 10.1016/j.jhep.2012.10.031]
15 **Lee BY**, Han JA, Im JS, Morrone A, Johung K, Goodwin EC, Kleijer WJ, DiMaio D, Hwang ES. Senescence-associated beta-galactosidase is lysosomal beta-galactosidase. *Aging Cell* 2006; **5**: 187-195 [PMID: 16626397 DOI: 10.1111/j.1474-9726.2006.00199.x]
16 **Debacq-Chainiaux F**, Erusalimsky JD, Campisi J, Toussaint O. Protocols to detect senescence-associated beta-galactosidase (SA-betagal) activity, a biomarker of senescent cells in culture and in vivo. *Nat Protoc* 2009; **4**: 1798-1806 [PMID: 20010931 DOI: 10.1038/nprot.2009.191]
17 **Ito Y**, Hoare M, Narita M. Spatial and Temporal Control of Senescence. *Trends Cell Biol* 2017; **27**: 820-832 [PMID: 28822679 DOI: 10.1016/j.tcb.2017.07.004]
18 **Lu T**, Finkel T. Free radicals and senescence. *Exp Cell Res* 2008; **314**: 1918-1922 [PMID: 18282568 DOI: 10.1016/j.yexcr.2008.01.011]
19 **Chen Q**, Fischer A, Reagan JD, Yan LJ, Ames BN. Oxidative DNA damage and senescence of human diploid fibroblast cells. *Proc Natl Acad Sci U S A* 1995; **92**: 4337-4341 [PMID: 7753808]
20 **Sherr CJ**, McCormick F. The RB and p53 pathways in cancer. *Cancer Cell* 2002; **2**: 103-112 [PMID: 12204530]
21 **Wei W**, Herbig U, Wei S, Dutriaux A, Sedivy JM. Loss of retinoblastoma but not p16 function allows bypass of replicative senescence in human fibroblasts. *EMBO Rep* 2003; **4**: 1061-1066 [PMID: 14566323 DOI: 10.1038/sj.embor.embor7400001]

22 **Webley K**, Bond JA, Jones CJ, Blaydes JP, Craig A, Hupp T, Wynford-Thomas D. Posttranslational modifications of p53 in replicative senescence overlapping but distinct from those induced by DNA damage. *Mol Cell Biol* 2000; **20**: 2803-2808 [PMID: 10733583]
23 **Li T**, Kon N, Jiang L, Tan M, Ludwig T, Zhao Y, Baer R, Gu W. Tumor suppression in the absence of p53-mediated cell-cycle arrest, apoptosis, and senescence. *Cell* 2012; **149**: 1269-1283 [PMID: 22682249 DOI: 10.1016/j.cell.2012.04.026]
24 **Aliouat-Denis CM**, Dendouga N, Van den Wyngaert I, Goehlmann H, Steller U, van de Weyer I, Van Slycken N, Andries L, Kass S, Luyten W, Janicot M, Vialard JE. p53-independent regulation of p21Waf1/Cip1 expression and senescence by Chk2. *Mol Cancer Res* 2005; **3**: 627-634 [PMID: 16317088 DOI: 10.1158/1541-7786.MCR-05-0121]
25 **Leinicke JA**, Longshore S, Wakeman D, Guo J, Warner BW. Regulation of retinoblastoma protein (Rb) by p21 is critical for adaptation to massive small bowel resection. *J Gastrointest Surg* 2012; **16**: 148-55; discussion 155 [PMID: 22042567 DOI: 10.1007/s11605-011-1747-8]
26 **Sage J**, Mulligan GJ, Attardi LD, Miller A, Chen S, Williams B, Theodorou E, Jacks T. Targeted disruption of the three Rb-related genes leads to loss of G(1) control and immortalization. *Genes Dev* 2000; **14**: 3037-3050 [PMID: 11114892]
27 **Ying H**, Xiao ZX. Targeting retinoblastoma protein for degradation by proteasomes. *Cell Cycle* 2006; **5**: 506-508 [PMID: 16552188 DOI: 10.4161/cc.5.5.2515]
28 **Dyson N**. The regulation of E2F by pRB-family proteins. *Genes Dev* 1998; **12**: 2245-2262 [PMID: 9694791]
29 **Okamoto K**, Kitabayashi I, Taya Y. KAP1 dictates p53 response induced by chemotherapeutic agents via Mdm2 interaction. *Biochem Biophys Res Commun* 2006; **351**: 216-222 [PMID: 17056014 DOI: 10.1016/j.bbrc.2006.10.022]
30 **Xing C**, Xie H, Zhou L, Zhou W, Zhang W, Ding S, Wei B, Yu X, Su R, Zheng S. Cyclin-dependent kinase inhibitor 3 is overexpressed in hepatocellular carcinoma and promotes tumor cell proliferation. *Biochem Biophys Res Commun* 2012; **420**: 29-35 [PMID: 22390936 DOI: 10.1016/j.bbrc.2012.02.107]
31 **Massagué J**. The transforming growth factor-beta family. *Annu Rev Cell Biol* 1990; **6**: 597-641 [PMID: 2177343 DOI: 10.1146/annurev.cb.06.110190.003121]
32 **Laiho M**, DeCaprio JA, Ludlow JW, Livingston DM, Massagué J. Growth inhibition by TGF-beta linked to suppression of retinoblastoma protein phosphorylation. *Cell* 1990; **62**: 175-185 [PMID: 2163767]
33 **Hannon GJ**, Beach D. p15INK4B is a potential effector of TGF-beta-induced cell cycle arrest. *Nature* 1994; **371**: 257-261 [PMID: 8078588 DOI: 10.1038/371257a0]
34 **Herranz N**, Gil J. Mechanisms and functions of cellular senescence. *J Clin Invest* 2018; **128**: 1238-1246 [PMID: 29608137 DOI: 10.1172/JCI95148]
35 **Freund A**, Orjalo AV, Desprez PY, Campisi J. Inflammatory networks during cellular senescence: causes and consequences. *Trends Mol Med* 2010; **16**: 238-246 [PMID: 20444648 DOI: 10.1016/j.molmed.2010.03.003]
36 **Sagiv A**, Krizhanovsky V. Immunosurveillance of senescent cells: the bright side of the senescence program. *Biogerontology* 2013; **14**: 617-628 [PMID: 24114507 DOI: 10.1007/s10522-013-9473-0]
37 **Acosta JC**, O'Loghlen A, Banito A, Guijarro MV, Augert A, Raguz S, Fumagalli M, Da Costa M, Brown C, Popov N, Takatsu Y, Melamed J, d'Adda di Fagagna F, Bernard D, Hernando E, Gil J. Chemokine signaling via the CXCR2 receptor reinforces senescence. *Cell* 2008; **133**: 1006-1018 [PMID: 18555777 DOI: 10.1016/j.cell.2008.03.038]
38 **Kuilman T**, Michaloglou C, Vredeveld LC, Douma S, van Doorn R, Desmet CJ, Aarden LA, Mooi WJ, Peeper DS. Oncogene-induced senescence relayed by an interleukin-dependent inflammatory network. *Cell* 2008; **133**: 1019-1031 [PMID: 18555778 DOI: 10.1016/j.cell.2008.03.039]
39 **Hoare M**, Ito Y, Kang TW, Weekes MP, Matheson NJ, Patten DA, Shetty S, Parry AJ, Menon S, Salama R, Antrobus R, Tomimatsu K, Howat W, Lehner PJ, Zender L, Narita M. NOTCH1 mediates a switch between two distinct secretomes during senescence. *Nat Cell Biol* 2016; **18**: 979-992 [PMID: 27525720 DOI: 10.1038/ncb3397]
40 **Dou Z**, Ghosh K, Vizioli MG, Zhu J, Sen P, Wangensteen KJ, Simithy J, Lan Y, Lin Y, Zhou Z, Capell BC, Xu C, Xu M, Kieckhaefer JE, Jiang T, Shoshkes-Carmel M, Tanim KMAA, Barber GN, Seykora JT, Millar SE, Kaestner KH, Garcia BA, Adams PD, Berger SL. Cytoplasmic chromatin triggers inflammation in senescence and cancer. *Nature* 2017; **550**: 402-406 [PMID: 28976970 DOI: 10.1038/nature24050]
41 **Li T**, Chen ZJ. The cGAS-cGAMP-STING pathway connects DNA damage to inflammation, senescence, and cancer. *J Exp Med* 2018; **215**: 1287-1299 [PMID: 29622565 DOI: 10.1084/jem.20180139]
42 **Barber GN**. STING: infection, inflammation and cancer. *Nat Rev Immunol* 2015; **15**: 760-770 [PMID: 26603901 DOI: 10.1038/nri3921]
43 **Glück S**, Guey B, Gulen MF, Wolter K, Kang TW, Schmacke NA, Bridgeman A, Rehwinkel J, Zender L, Ablasser A. Innate immune sensing of cytosolic chromatin fragments through cGAS promotes senescence. *Nat Cell Biol* 2017; **19**: 1061-1070 [PMID: 28759028 DOI: 10.1038/ncb3586]
44 **De Cecco M**, Ito T, Petrashen AP, Elias AE, Skvir NJ, Criscione SW, Caligiana A, Brocculi G, Adney EM, Boeke JD, Le O, Beauséjour C, Ambati J, Ambati K, Simon M, Seluanov A, Gorbunova V, Slagboom PE, Helfand SL, Neretti N, Sedivy JM. L1 drives IFN in senescent cells and promotes age-associated inflammation. *Nature* 2019; **566**: 73-78 [PMID: 30728521 DOI: 10.1038/s41586-018-0784-9]
45 **Verdin E**. NAD in aging, metabolism, and neurodegeneration. *Science* 2015; **350**: 1208-1213 [PMID: 26785480 DOI: 10.1126/science.aac4854]
46 **Garten A**, Schuster S, Penke M, Gorski T, de Giorgis T, Kiess W. Physiological and pathophysiological roles of NAMPT and NAD metabolism. *Nat Rev Endocrinol* 2015; **11**: 535-546 [PMID: 26215259 DOI: 10.1038/nrendo.2015.117]
47 **Nacarelli T**, Lau L, Fukumoto T, Zundell J, Fatkhutdinov N, Wu S, Aird KM, Iwasaki O, Kossenkov AV, Schultz D, Noma KI, Baur JA, Schug Z, Tang HY, Speicher DW, David G, Zhang R. NAD^+ metabolism governs the proinflammatory senescence-associated secretome. *Nat Cell Biol* 2019; **21**: 397-407 [PMID: 30778219 DOI: 10.1038/s41556-019-0287-4]
48 **Narita M**, Narita M, Krizhanovsky V, Nuñez S, Chicas A, Hearn SA, Myers MP, Lowe SW. A novel role for high-mobility group a proteins in cellular senescence and heterochromatin formation. *Cell* 2006; **126**: 503-514 [PMID: 16901784 DOI: 10.1016/j.cell.2006.05.052]
49 **Davalos AR**, Kawahara M, Malhotra GK, Schaum N, Huang J, Ved U, Beausejour CM, Coppe JP, Rodier F, Campisi J. p53-dependent release of Alarmin HMGB1 is a central mediator of senescent phenotypes. *J Cell Biol* 2013; **201**: 613-629 [PMID: 23649808 DOI: 10.1083/jcb.201206006]
50 **Yang H**, Wang H, Ren J, Chen Q, Chen ZJ. cGAS is essential for cellular senescence. *Proc Natl Acad Sci*

USA 2017; **114**: E4612-E4620 [PMID: 28533362 DOI: 10.1073/pnas.1705499114]
51 **Tasdemir N**, Banito A, Roe JS, Alonso-Curbelo D, Camiolo M, Tschaharganeh DF, Huang CH, Aksoy O, Bolden JE, Chen CC, Fennell M, Thapar V, Chicas A, Vakoc CR, Lowe SW. BRD4 Connects Enhancer Remodeling to Senescence Immune Surveillance. *Cancer Discov* 2016; **6**: 612-629 [PMID: 27099234 DOI: 10.1158/2159-8290.CD-16-0217]
52 **Laberge RM**, Sun Y, Orjalo AV, Patil CK, Freund A, Zhou L, Curran SC, Davalos AR, Wilson-Edell KA, Liu S, Limbad C, Demaria M, Li P, Hubbard GB, Ikeno Y, Javors M, Desprez PY, Benz CC, Kapahi P, Nelson PS, Campisi J. MTOR regulates the pro-tumorigenic senescence-associated secretory phenotype by promoting IL1A translation. *Nat Cell Biol* 2015; **17**: 1049-1061 [PMID: 26147250 DOI: 10.1038/ncb3195]
53 **Young AR**, Narita M, Ferreira M, Kirschner K, Sadaie M, Darot JF, Tavaré S, Arakawa S, Shimizu S, Watt FM, Narita M. Autophagy mediates the mitotic senescence transition. *Genes Dev* 2009; **23**: 798-803 [PMID: 19279323 DOI: 10.1101/gad.519709]
54 **Kang HT**, Lee KB, Kim SY, Choi HR, Park SC. Autophagy impairment induces premature senescence in primary human fibroblasts. *PLoS One* 2011; **6**: e23367 [PMID: 21858089 DOI: 10.1371/journal.pone.0023367]
55 **García-Prat L**, Martínez-Vicente M, Perdiguero E, Ortet L, Rodríguez-Ubreva J, Rebollo E, Ruiz-Bonilla V, Gutarra S, Ballestar E, Serrano AL, Sandri M, Muñoz-Cánoves P. Autophagy maintains stemness by preventing senescence. *Nature* 2016; **529**: 37-42 [PMID: 26738589 DOI: 10.1038/nature16187]
56 **Kang C**, Xu Q, Martin TD, Li MZ, Demaria M, Aron L, Lu T, Yankner BA, Campisi J, Elledge SJ. The DNA damage response induces inflammation and senescence by inhibiting autophagy of GATA4. *Science* 2015; **349**: aaa5612 [PMID: 26404840 DOI: 10.1126/science.aaa5612]
57 **Cao X**, Li M. A New Pathway for Senescence Regulation. *Genomics Proteomics Bioinformatics* 2015; **13**: 333-335 [PMID: 26777575 DOI: 10.1016/j.gpb.2015.11.002]
58 **Perlman H**, Suzuki E, Simonson M, Smith RC, Walsh K. GATA-6 induces p21(Cip1) expression and G1 cell cycle arrest. *J Biol Chem* 1998; **273**: 13713-13718 [PMID: 9593712]
59 **Zhang Z**, Yao Z, Zhao S, Shao J, Chen A, Zhang F, Zheng S. Interaction between autophagy and senescence is required for dihydroartemisinin to alleviate liver fibrosis. *Cell Death Dis* 2017; **8**: e2886 [PMID: 28617435 DOI: 10.1038/cddis.2017.255]
60 **Ferreira-Gonzalez S**, Lu WY, Raven A, Dwyer B, Man TY, O'Duibhir E, Lewis PJS, Campana L, Kendall TJ, Bird TG, Tarrats N, Acosta JC, Boulter L, Forbes SJ. Paracrine cellular senescence exacerbates biliary injury and impairs regeneration. *Nat Commun* 2018; **9**: 1020 [PMID: 29523787 DOI: 10.1038/s41467-018-03299-5]
61 **Paradis V**, Youssef N, Dargère D, Bâ N, Bonvoust F, Deschatrette J, Bedossa P. Replicative senescence in normal liver, chronic hepatitis C, and hepatocellular carcinomas. *Hum Pathol* 2001; **32**: 327-332 [PMID: 11274643 DOI: 10.1053/hupa.2001.22747]
62 **Wiemann SU**, Satyanarayana A, Tsahuridu M, Tillmann HL, Zender L, Klempnauer J, Flemming P, Franco S, Blasco MA, Manns MP, Rudolph KL. Hepatocyte telomere shortening and senescence are general markers of human liver cirrhosis. *FASEB J* 2002; **16**: 935-942 [PMID: 12087054 DOI: 10.1096/fj.01-0977com]
63 **Gutierrez-Reyes G**, del Carmen Garcia de Leon M, Varela-Fascinetto G, Valencia P, Pérez Tamayo R, Rosado CG, Labonne BF, Rochilin NM, Garcia RM, Valadez JA, Latour GT, Corona DL, Diaz GR, Zlotnik A, Kershenobich D. Cellular senescence in livers from children with end stage liver disease. *PLoS One* 2010; **5**: e10231 [PMID: 20422055 DOI: 10.1371/journal.pone.0010231]
64 **Frey N**, Venturelli S, Zender L, Bitzer M. Cellular senescence in gastrointestinal diseases: from pathogenesis to therapeutics. *Nat Rev Gastroenterol Hepatol* 2018; **15**: 81-95 [PMID: 29184185 DOI: 10.1038/nrgastro.2017.146]
65 **Krizhanovsky V**, Yon M, Dickins RA, Hearn S, Simon J, Miething C, Yee H, Zender L, Lowe SW. Senescence of activated stellate cells limits liver fibrosis. *Cell* 2008; **134**: 657-667 [PMID: 18724938 DOI: 10.1016/j.cell.2008.06.049]
66 **Demaria M**, Ohtani N, Youssef SA, Rodier F, Toussaint W, Mitchell JR, Laberge RM, Vijg J, Van Steeg H, Dollé ME, Hoeijmakers JH, de Bruin A, Hara E, Campisi J. An essential role for senescent cells in optimal wound healing through secretion of PDGF-AA. *Dev Cell* 2014; **31**: 722-733 [PMID: 25499914 DOI: 10.1016/j.devcel.2014.11.012]
67 **Chène P**. Inhibiting the p53-MDM2 interaction: an important target for cancer therapy. *Nat Rev Cancer* 2003; **3**: 102-109 [PMID: 12563309 DOI: 10.1038/nrc991]
68 **Zhou T**, Wu N, Meng F, Venter J, Giang TK, Francis H, Kyritsi K, Wu C, Franchitto A, Alvaro D, Marzioni M, Onori P, Mancinelli R, Gaudio E, Glaser S, Alpini G. Knockout of secretin receptor reduces biliary damage and liver fibrosis in Mdr2$^{-/-}$ mice by diminishing senescence of cholangiocytes. *Lab Invest* 2018; **98**: 1449-1464 [PMID: 29977037 DOI: 10.1038/s41374-018-0093-9]
69 **Wan Y**, Meng F, Wu N, Zhou T, Venter J, Francis H, Kennedy L, Glaser T, Bernuzzi F, Invernizzi P, Glaser S, Huang Q, Alpini G. Substance P increases liver fibrosis by differential changes in senescence of cholangiocytes and hepatic stellate cells. *Hepatology* 2017; **66**: 528-541 [PMID: 28256736 DOI: 10.1002/hep.29138]
70 **McDaniel K**, Meng F, Wu N, Sato K, Venter J, Bernuzzi F, Invernizzi P, Zhou T, Kyritsi K, Wan Y, Huang Q, Onori P, Francis H, Gaudio E, Glaser S, Alpini G. Forkhead box A2 regulates biliary heterogeneity and senescence during cholestatic liver injury in mice‡. *Hepatology* 2017; **65**: 544-559 [PMID: 27639079 DOI: 10.1002/hep.28831]
71 **Liu Y**, He J, Ji S, Wang Q, Pu H, Jiang T, Meng L, Yang X, Ji J. Comparative studies of early liver dysfunction in senescence-accelerated mouse using mitochondrial proteomics approaches. *Mol Cell Proteomics* 2008; **7**: 1737-1747 [PMID: 18515266 DOI: 10.1074/mcp.M800109-MCP200]
72 **Clouston AD**, Powell EE, Walsh MJ, Richardson MM, Demetris AJ, Jonsson JR. Fibrosis correlates with a ductular reaction in hepatitis C: roles of impaired replication, progenitor cells and steatosis. *Hepatology* 2005; **41**: 809-818 [PMID: 15793848 DOI: 10.1002/hep.20650]
73 **Cieslak KP**, Baur O, Verheij J, Bennink RJ, van Gulik TM. Liver function declines with increased age. *HPB (Oxford)* 2016; **18**: 691-696 [PMID: 27485064 DOI: 10.1016/j.hpb.2016.05.011]
74 **Schmucker DL**. Age-related changes in liver structure and function: Implications for disease? *Exp Gerontol* 2005; **40**: 650-659 [PMID: 16102930 DOI: 10.1016/j.exger.2005.06.009]
75 **Zhang HJ**, Xu L, Drake VJ, Xie L, Oberley LW, Kregel KC. Heat-induced liver injury in old rats is associated with exaggerated oxidative stress and altered transcription factor activation. *FASEB J* 2003; **17**: 2293-2295 [PMID: 14500545 DOI: 10.1096/fj.03-0139fje]
76 **Intano GW**, Cho EJ, McMahan CA, Walter CA. Age-related base excision repair activity in mouse brain

and liver nuclear extracts. *J Gerontol A Biol Sci Med Sci* 2003; **58**: 205-211 [PMID: 12634285 DOI: 10.1093/gerona/58.3.b205]

77 **Xiao M**, Chen W, Wang C, Wu Y, Zhu S, Zeng C, Cai Y, Liu C, He Z. Senescence and cell death in chronic liver injury: roles and mechanisms underlying hepatocarcinogenesis. *Oncotarget* 2017; **9**: 8772-8784 [PMID: 29492237 DOI: 10.18632/oncotarget.23622]

78 **Rudolph KL**, Chang S, Millard M, Schreiber-Agus N, DePinho RA. Inhibition of experimental liver cirrhosis in mice by telomerase gene delivery. *Science* 2000; **287**: 1253-1258 [PMID: 10678830 DOI: 10.1126/science.287.5456.1253]

79 **Teoh N**, Pyakurel P, Dan YY, Swisshelm K, Hou J, Mitchell C, Fausto N, Gu Y, Farrell G. Induction of p53 renders ATM-deficient mice refractory to hepatocarcinogenesis. *Gastroenterology* 2010; **138**: 1155-1165.e1-2 [PMID: 19919837 DOI: 10.1053/j.gastro.2009.11.008]

80 **Kang TW**, Yevsa T, Woller N, Hoenicke L, Wuestefeld T, Dauch D, Hohmeyer A, Gereke M, Rudalska R, Potapova A, Iken M, Vucur M, Weiss S, Heikenwalder M, Khan S, Gil J, Bruder D, Manns M, Schirmacher P, Tacke F, Ott M, Luedde T, Longerich T, Kubicka S, Zender L. Senescence surveillance of pre-malignant hepatocytes limits liver cancer development. *Nature* 2011; **479**: 547-551 [PMID: 22080947 DOI: 10.1038/nature10599]

81 **Eggert T**, Wolter K, Ji J, Ma C, Yevsa T, Klotz S, Medina-Echeverz J, Longerich T, Forgues M, Reisinger F, Heikenwalder M, Wang XW, Zender L, Greten TF. Distinct Functions of Senescence-Associated Immune Responses in Liver Tumor Surveillance and Tumor Progression. *Cancer Cell* 2016; **30**: 533-547 [PMID: 27728804 DOI: 10.1016/j.ccell.2016.09.003]

82 **Mudbhary R**, Hoshida Y, Chernyavskaya Y, Jacob V, Villanueva A, Fiel MI, Chen X, Kojima K, Thung S, Bronson RT, Lachenmayer A, Revill K, Alsinet C, Sachidanandam R, Desai A, SenBanerjee S, Ukomadu C, Llovet JM, Sadler KC. UHRF1 overexpression drives DNA hypomethylation and hepatocellular carcinoma. *Cancer Cell* 2014; **25**: 196-209 [PMID: 24486181 DOI: 10.1016/j.ccr.2014.01.003]

83 **Iannello A**, Thompson TW, Ardolino M, Lowe SW, Raulet DH. p53-dependent chemokine production by senescent tumor cells supports NKG2D-dependent tumor elimination by natural killer cells. *J Exp Med* 2013; **210**: 2057-2069 [PMID: 24043758 DOI: 10.1084/jem.20130783]

84 **Xue W**, Zender L, Miething C, Dickins RA, Hernando E, Krizhanovsky V, Cordon-Cardo C, Lowe SW. Senescence and tumour clearance is triggered by p53 restoration in murine liver carcinomas. *Nature* 2007; **445**: 656-660 [PMID: 17251933 DOI: 10.1038/nature05529]

85 **Harada K**, Furubo S, Ozaki S, Hiramatsu K, Sudo Y, Nakanuma Y. Increased expression of WAF1 in intrahepatic bile ducts in primary biliary cirrhosis relates to apoptosis. *J Hepatol* 2001; **34**: 500-506 [PMID: 11394648]

86 **Sasaki M**, Ikeda H, Yamaguchi J, Miyakoshi M, Sato Y, Nakanuma Y. Bile ductular cells undergoing cellular senescence increase in chronic liver diseases along with fibrous progression. *Am J Clin Pathol* 2010; **133**: 212-223 [PMID: 20093230 DOI: 10.1309/AJCPWMX47TREYWZG]

87 **Fabregat I**, Moreno-Càceres J, Sánchez A, Dooley S, Dewidar B, Giannelli G, Ten Dijke P; IT-LIVER Consortium. TGF-β signalling and liver disease. *FEBS J* 2016; **283**: 2219-2232 [PMID: 26807763 DOI: 10.1111/febs.13665]

88 **Fickert P**, Wagner M. Biliary bile acids in hepatobiliary injury - What is the link? *J Hepatol* 2017; **67**: 619-631 [PMID: 28712691 DOI: 10.1016/j.jhep.2017.04.026]

89 **Khambu B**, Li T, Yan S, Yu C, Chen X, Goheen M, Li Y, Lin J, Cummings OW, Lee YA, Friedman S, Dong Z, Feng GS, Wu S, Yin XM. Hepatic Autophagy Deficiency Compromises Farnesoid X Receptor Functionality and Causes Cholestatic Injury. *Hepatology* 2019; **69**: 2196-2213 [PMID: 30520052 DOI: 10.1002/hep.30407]

90 **Panebianco C**, Oben JA, Vinciguerra M, Pazienza V. Senescence in hepatic stellate cells as a mechanism of liver fibrosis reversal: a putative synergy between retinoic acid and PPAR-gamma signalings. *Clin Exp Med* 2017; **17**: 269-280 [PMID: 27655446 DOI: 10.1007/s10238-016-0438-x]

91 **Yoshimoto S**, Loo TM, Atarashi K, Kanda H, Sato S, Oyadomari S, Iwakura Y, Oshima K, Morita H, Hattori M, Honda K, Ishikawa Y, Hara E, Ohtani N. Obesity-induced gut microbial metabolite promotes liver cancer through senescence secretome. *Nature* 2013; **499**: 97-101 [PMID: 23803760 DOI: 10.1038/nature12347]

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MINIREVIEWS

Role of endoscopic ultrasound in the screening and follow-up of high-risk individuals for familial pancreatic cancer

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Abstract

Managing familial pancreatic cancer (FPC) is challenging for gastroenterologists, surgeons and oncologists. High-risk individuals (HRI) for pancreatic cancer (PC) (FPC or with germline mutations) are a heterogeneous group of subjects with a theoretical lifetime cumulative risk of PC over 5%. Screening is mainly based on annual magnetic resonance imaging (MRI) and endoscopic ultrasound (EUS). The goal of screening is to identify early-stage operable cancers or high-risk precancerous lesions (pancreatic intraepithelial neoplasia or intraductal papillary mucinous neoplasms with high-grade dysplasia). In the literature, target lesions are identified in 2%-5% of HRI who undergo screening. EUS appears to provide better identification of small solid lesions (0%-46% of HRI) and chronic-pancreatitis-like parenchymal changes (14%-77% of HRI), while MRI is probably the best modality to identify small cystic lesions (13%-49% of HRI). There are no specific studies in HRI on the use of contrast-enhanced harmonic EUS. EUS can also be used to obtain tissue samples. Nevertheless, there is still limited evidence on the accuracy of imaging procedures used for screening or agreement on which patients to treat. The cost-effectiveness of screening is also unclear. Certain new EUS-related techniques, such as searching for DNA abnormalities or protein markers in pancreatic fluid, appear to be promising.

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Core tip: High-risk individuals (HRI) for pancreatic cancer have a lifetime cumulative risk of this disorder of over 5%. The goal of screening is to identify operable cancers or precancerous lesions. Endoscopic ultrasound (EUS) appears to better identify solid lesions (0%-46% of HRI) and chronic-pancreatitis-like parenchymal changes (14%-77%), and magnetic resonance imaging to better identify small cysts (13%-49%). EUS is used to obtain tissue samples. There are no specific studies on contrast-enhanced harmonic EUS in HRI. There is limited evidence on the accuracy of imaging used for screening or agreement on which patients to treat. The cost-effectiveness of screening is also unclear. New EUS-related techniques (identifying DNA abnormalities or protein markers) appear to be promising.

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INTRODUCTION

In the past few decades, the incidence of pancreatic cancer (PC) has continuously increased, while its prognosis remains poor, with a 5-year survival rate < 10% for all stages analysed together. PC is expected to become the second leading cause of cancer-related death in the United States in 2030[1]. Early-stage surgical resection is the only potentially curative treatment that increases survival. However, complete surgical resection can only be performed in a minority of patients, since 80% of patients have metastatic or locoregionally advanced disease at diagnosis[1-3].

Five to 10% of PCs are considered to be inherited[4,5]. While pathogenic germline mutations in specific genes have been associated with an increased risk of PC (from 4% to 40%), a causal germline mutation is identified in fewer than 20% of these families[6-10]. Several gene abnormalities and related hereditary syndromes have been associated with an increased risk of PC: *BRCA1* and *BRCA2* (hereditary breast ovarian cancer syndrome), *PALB2* and the genes involved in the Fanconi pathway, *CDKN2A* (familial atypical multiple mole melanoma: FAMMM), the genes involved in Lynch syndrome (mainly *MLH1*, *MSH2*, *MSH6*, and *PMS2*), *PRSS1* (hereditary pancreatitis), *STK11* (Peutz-Jeghers syndrome), *TP53* (Li-Fraumeni syndrome), *APC* (familial adenomatous polyposis), and *ATM* (ataxia telangiectasia)[4,9,11-16]. In the remaining 85% of families with no identified germline mutation, familial pancreatic cancer (FPC) is defined by the occurrence of PC in ≥ 2 first-degree relatives or in ≥ 3 relatives whatever the degree of relationship on the same side of the family[4,17].

Pancreatic screening is recommended in high-risk individuals (HRI) to identify early (pre)malignant pancreatic lesions and propose surgical resection with curative intent. The purpose of this screening is to reduce mortality related to PC. While numerous large and retrospective studies have reported on the short-term outcomes of pancreatic screening in HRI, follow-up was generally limited, and hence the magnitude of benefit of pancreatic screening in terms of curative potential remains currently unknown[11,12,17-19]. Additionally, although screening is usually based on yearly magnetic resonance imaging (MRI) and endoscopic ultrasound (EUS), the role of the latter has not been clearly defined. Our goal was to comprehensively review current data on pancreatic screening and follow-up of HRI, with an emphasis on the role of EUS.

OBJECTIVES AND CHALLENGES OF PANCREATIC SCREENING IN HIGH-RISK INDIVIDUALS

The goal of screening in HRI (history of FPC or pathogenic germline mutations +/- family history of PC) is to reduce PC-related mortality by identifying morphological abnormalities that suggest the development of PC at an early and potentially curative stage[11,17-19]. In a meta-analysis from our group including all HRI treated by surgery reported in the literature, patients without invasive PC who underwent resection of premalignant lesions had no postoperative recurrence compared to those with invasive PC, and their 3-year overall survival was 100% *vs* 34.6%, respectively ($P < 0.001$)[11]. The lifetime risk of PC in HRI (with germline mutations or FPC) is estimated to be between 1% and 50% depending on the underlying predisposition and the number of affected relatives[12,17,20]. Table 1 presents the risk by mutations and their frequency in inherited cancers. Thus, HRI are theoretically good candidates for pancreatic screening.

The first challenge of PC screening is the effective identification of good candidates, specifically, individuals with a theoretical risk threshold, arbitrarily set at 5%, of developing PC in their lifetime. Signoretti *et al*[18,21] have shown that the identification of (pre)malignant lesions varies depending on the genetic subgroup (3% in familial PC, 5% in FAMM syndrome, 6.3% in hereditary breast/ovarian cancer, and 12.2% in Peutz-Jeghers syndrome), while it was 42% in patients with hereditary pancreatitis who have the PRSS1 mutation. Corral *et al*[19] estimated that 135 patients needed to be screened to successfully identify 1 patient with a target lesion (high-risk lesion or PC) (95%CI: 88-303). This low rate was highly questionable, however, due to the very short follow-up period (3.3 years on average) reported in the studies[22]. Indeed, it contrasts with the delay that was estimated for a premalignant lesion to transform into invasive cancer (11 years) and does not enable the drawing of conclusions regarding the global yield of pancreatic screening in HRI.

Relevant imaging pancreatic abnormalities are identified at imaging in approximately 50% of HRI, but this figure is difficult to interpret as there have been too few correlations of these imaging abnormalities with pathological examination due to the limited number of operated patients[11,18,23]. Another challenge of pancreatic screening is to identify and use the most appropriate screening techniques. Ideally, this would be the least invasive and reproducible technique that identifies the greatest number of premalignant lesions and that is the most acceptable for the patient.

The ultimate goal of this approach is to propose surgical resection of premalignant lesions [such as pancreatic intraepithelial neoplasia (PanIN) or intraductal papillary mucinous neoplasms (IPMN) with high-grade dysplasia (HGD)] or even early-stage invasive PC, which are found in approximately 3%-5% of HRI[11,19]. Finally, identifying lesions at high risk of (pre)malignancy and operating neither too early (low-grade dysplasia) nor too late (advanced PC) is challenging[17]. We recently found that an indication for prophylactic pancreatectomy was appropriate (based on identification of HGD or invasive PC) in 42.2% of surgically treated HRI[11]. The factors predicting surgical appropriateness were age > 50 years, presence of a germline mutation and the presence of high-risk radiological pancreatic abnormalities (the presence of "worrisome features", "high-risk stigmata of malignancy", or a solid pancreatic mass)[11].

WHAT IS THE BEST SCREENING MODALITY FOR HIGH-RISK INDIVIDUALS?

In the past two decades, management of HRI has evolved and varies from one country to another. Screening should be performed in multidisciplinary teams in referral centres, which have more experience and expertise in screening methods (i.e., EUS and MRI) and in the treatment of invasive PC[17]. A recent meta-analysis estimated that the annual prevalence of high-risk lesions (early invasive PC, IPMN, or PanIN with HGD) detected in HRI was 3.3%, corresponding to 5/1000 person-years during follow-up and an individual probability of 0.5% per year[18].

The screening of HRI is mainly based on pancreatic morphological imaging [computed tomography (CT) scan, MRI and EUS][11,12,18,19]. For a long time, many studies have suggested that EUS might provide better detection of small solid lesions, while MRI can identify small cystic lesions[24-27]. In the study by Canto *et al*[25] in 216 HRI, EUS, MRI and CT scan detected pancreatic abnormalities (cysts, solid lesions or chronic pancreatitis) in 42.6%, 33.3% and 11% of patients, respectively. This corresponded to a sensitivity of 93% for EUS for the detection of solid lesions smaller than 2 cm compared to 53% and 67% for CT scan and MRI, respectively[25]. Harinck *et*

Table 1 Risk of pancreatic cancer based on genetic mutations

Condition	Gene	Relative risk of PC compared to the general population	Risk of pancreatic cancer at 70 yr (%)	% Among inherited cancers
No family history		1	0.5-1	?
2 first degree relatives with PC	Unknown	5-7	5-12	80-85
3 first degree relatives with PC	Unknown	32	40	
Hereditary breast ovarian cancer syndrome	*BRCA1*	2-4	3-4	1-5
	BRCA2	2-10	4-5	5-20
Genetic pancreatitis	PRSS1	50-80	40-55	1-4
FAMMM	*CDKN2A*	10-25	5-25	2-3
Peutz-Jeghers syndrome	*STK11*	100-130	30-40	1-3
Lynch syndrome	*MLH1*, *MSH2*, *MSH6*, *PMS2*	4-8	3-5	1-3

PC: pancreatic cancer; FAMMM: Familial atypical multiple mole melanoma.

al[27] performed a prospective comparison of EUS and MRI for the detection of clinically relevant pancreatic lesions at initial screening of 139 HRI. In this study, EUS and/or MRI detected pancreatic lesions in 6% of HRI: 2 solid tumours < 10 mm were only detected by EUS (1 invasive PC and 1 PanIN with low-grade dysplasia), and 25% of cysts were only detected by MRI[27]. Nevertheless, as all patients were not operated on, this study does not enable the evaluation of whether the lesions detected were all of pathological relevance. Table 2 reports the main characteristics of HRI screening techniques and imaging results in 16 published studies. Of note, MRI and CT scan protocols were not clearly described in most studies (*e.g.*, matrix size, contrast enhancement, MRI sequences), and the results of EUS are well known to be operator-dependent as well as classical radiological procedures[28,29]. Indeed, Topazian *et al*[28] report a low interobserver agreement for the interpretation of pancreatic EUS in HRI (Kappa < 0.4 except for cysts). This is probably due to the lack of specific training for EUS, the lack of a standardized collection chart and a specific learning curve. Although all of the abovementioned studies included operated patients, the methods of detection of the pancreatic abnormalities that determined the surgical procedure were not described in detail. Thus, while the precise value of EUS compared to the other modalities is probably high (it may find more (pre)malignant lesions), this is difficult to determine in the absence of a large study correlating anatomopathological specimens to EUS findings.

There are no approved biomarkers for the screening of PC in HRI. Only one study has reported the results of serum CA19-9 measurement for pancreatic screening in these patients. Twenty-seven out of 546 included patients (4.9%) had elevated CA19-9, and 5 (18.5%) of these had pancreatic lesions (1 PC, 2 IPMN, 1 PanIN, 1 neuroendocrine tumour). Nevertheless, the number of pancreatic lesions in the group with normal CA 19-9 levels was not reported[16]. CA19-9 is not recommended because of its low sensitivity and specificity[11,17-19,30].

The recent CAPS (International Cancer of the Pancreas Screening) consensus has suggested that annual MRI [including magnetic resonance cholangio-pancreatography (MRCP)] and EUS are the best imaging modalities for the detection of significant PC precursor lesions[6]. In summary, and as recommended by the recent statement by the American Society of Clinical Oncology, pancreatic screening and follow-up in HRI should be based on MRI and EUS as complementary tests for the detection of pancreatic lesions[17].

WHEN AND HOW OFTEN SHOULD SCREENING BE PERFORMED?

While pancreatic screening usually begins when HRI are 40 years old, or 10 years before the youngest index case[23], this has recently been challenged because pancreatic screening rarely reveals relevant lesions before the age of 50[4]. We also recently reported that the risk of HGD or invasive cancer was 3 times higher in HRI > 50 years old operated on for pancreatic lesions than in HRI < 50 years old[11].

Table 2 Main characteristics of high-risk individuals for pancreatic cancer in the different studies

First author/yr	Setting	High risk condition	Patients included	Modalities of screening	Imaging results	Patients operated on	Operated lesions seen only by the EUS	Histological features with cancer or HGD
Brentnall *et al*[37]	Prospective, monocentric	FPC	14	EUS	CPis: 10 (77%) Cystic lesio: ? Solid lesio: 6 (46%)	7	[1]NA	7 dysplasia
Rulyak *et al*[38]	Prospective, monocentric	FPC	35	EUS, ERCP	12 lesions	12	?	12 dysplasia
Kimmey *et al*[78]	Prospective, monocentric	FPC	46	EUS, ERCP	CPis: 24 (52%) Cystic lesion: ? Solid lesion: 12 (26%)	?	?	?
Canto *et al*[12]	Prospective, monocentric	FPC, PJS,	38	EUS first, +/- ERCP, CT, FNA	CPis: 17 (45%) Cystic lesion: ? Solid lesion: 12 (31%)	7	2/7 (1 PC, 1 PanIN3)	1 PC 1 PanIN3
Canto *et al*[23]	Prospective, monocentric	FPC, PJS,	78	EUS, CT +/- ERCP, FNA	CPis: 61 (78%) Cystic lesion: 9 (12%) Solid lesion: 8 (10%)	7	3/7 (1 PanIN 3, 2 PanIN1-2)	1 PC+IPMN 1 PanIN3 1PanIN3+IPMN
Poley *et al*[13]	Prospective, multicentric	FPC, PJS, FAMMM, FBOC, HP, LFS	44	EUS	CPis: 3 (7%) Cystic lesion: 7 (16%) Solid lesion: 3 (7%)	10	[1]NA	3 PC
Langer *et al*[15]	Prospective, multicentric	FPC, FAMMM	76	EUS + MRI	CPis: 17 (22%) Cystic lesion: 3 (4%) Solid lesion: 7 (9%)	7	5/7 (9/21 non operated lesions seen only in EUS)	0 PC or PanIN3 or IPMN with HGD
Verna *et al*[33]	Prospective, monocentric	FPC, FBOC	51	EUS *n* = 31 or MRI *n* = 33 +/- ERCP, FNA	CPis: 9 (29%) Cystic lesion: 12 (39%) Solid lesion: 2 (6%)	5	? (2 solid lesions in EUS and MRI)	1 PC 1 PanIN2
Ludwig *et al*[34]	Prospective, monocentric	FPC	109	MRCP or CT EUS, FNA	18 lesions	6	?	1 PC 2 MD IPMN 1 PanIN-3 1 PanIN-2
Schneider *et al*[40]	Prospective, monocentric	FPC, FAMMM	72	EUS, MRI	13 lesions	9	?	1 PC 1 PanIN-3
Zubarik *et al*[16]	Prospective, monocentric	FPC, PJS, BRCA2	27	CA19-9 +/- EUS (27/546)	5 lesions	5	[1]NA	1 PC
Canto *et al*[25]	Prospective, multicentric	FPC, FBOC, PJS	216	CT, EUS, MRI/MRCP	CPis: 54 (25%) Cystic lesion: 79 (36%) Solid lesion: 3 (1.4%)	5	? (20/216 non operated lesions seen only in EUS including 3 solid lesion)	2 MD-IPMN 1 IPMN+PanIN3
Al-Sukhni *et al*[32]	Prospective, monocentric	FPC, BRCA1/2, FAMMM, LKB1	252	MRI, +/- CT, EUS, ERCP	CPis: ? Cystic lesion: 80 (32%) Solid lesion: 3 (1.2%)	4		3 PC
Sud *et al*[14]	Prospective, monocentric	FPC, BRCA1/2, MMR, CDKN2A, LKB1, PRSS1	30	EUS +/- FNA	3 lesions	3	[1]NA	2 PC
Mocci *et al*[10]	Prospective, multicentric	FPC, CDKN2A, MMR, PRSS1	41	EUS, CT +/- MRI, FNA	CPis: 15 (37%) Cystic lesion:4 (10%) Solid lesion: 2 (5%)	1	[2]NA	1 NET 1 PanIN3
Vasen *et al*[31]	Prospective, multicentric	FPC, CDKN2A, BRCA 1/2	411	MRI, EUS, CT, FNA	CPis: ? Cystic lesion: 138 (34%) Solid lesion: 16 (4%)	30	2/30 (866 MRIs and 106 EUS performed)	16 PC 3 PanIN3 1 IPMN with high grade

Bartsch *et al*[4]	Prospective, multicentric	FPC, BRCA1/2, PALB2 234	MRI, EUS, CT, FNA	CPis: ? Cystic lesion: 125 (49%) Solid lesion: 9 (4%)	21 6/253 (2%) lesions (2 malignant and HGD)	?	2 PC 3 PanIN3 1 IPMN with HGD; multifocal PanIN2±BD-IPMN±AFL 1 NET 8 PanIN-2
Harinck *et al*[27]	Prospective, multicentric	FPC, BRCA1/2, CDKN2A, LKB1, TP53, MMR 139	MRI +/- EUS	CPis: 41 (30%) Cystic lesion: 67 (48%) Solid lesion: 2 (1.4%)	2	2/2 (1 PC, 1 PanIN-2)	1 PC 1 PanIN-2

[1]NA: Not applicable, because patients had only endoscopic ultrasound (EUS);
[2]NA: Not applicable, because patients had an EUS only if magnetic resonance imaging was abnormal. Three patients developed pancreatic cancer during the screening. CPis: Chronic-pancreatitis-like parenchymal changes; CT: Computed tomography; EUS: Endoscopic ultrasound; FNA: Fine needle aspiration; FPC: Familial pancreatic cancer; IPMN: Intraductal papillary mucinous neoplasm; MRI: Magnetic resonance imaging; PanIN: Pancreatic intraneoplasia; PC: Pancreatic cancer; ERCP: Endoscopic retrograde cholangiopancreaticography; HGD: High-grade dysplasia.

It is important to note that the majority of relevant pancreatic lesions (> 50% of cysts and solid tumours) are identified at the first screening rather than during follow-up[23,31,32]. The frequency of subsequent follow-up examinations varies depending on different studies and recommendations[4,30-34]. Several multicenter studies have compared different surveillance protocols (*e.g.*, annual MRI and EUS or annual MRI with EUS every 3 years) and did not find any difference in terms of number of diagnosed lesions[4,31]. However, these studies compared the total number of diagnosed lesions, whereas only the number of (pre)malignant lesions would have had clinical relevance. Nevertheless, and unless demonstrated otherwise, it seems reasonable to perform one MRI and one EUS examination per year in HRI[11,19,31,32].

Life-long pancreatic follow-up is recommended in HRI, at least as long as patients remain fit for surgery, without severe comorbidities. In fact, there are no strong data to confirm when follow-up should be stopped[17,23]. Furthermore, the prognosis in another group at risk of PC (patients with IPMN) was poorer in patients who stopped surveillance after 5 years of surveillance, which advocates for prolonged surveillance[35].

PLACE OF ENDOSCOPIC ULTRASOUND FOR PANCREATIC SCREENING OF HIGH-RISK INDIVIDUALS

Pancreatic lesions found in endoscopic ultrasound

EUS pancreatic screening may identify pancreatic abnormalities in 19% to 79% of HRI (13–16, 25, 31, 34, 35, 37–40) (Table 2). These pancreatic "abnormalities" include cystic lesions (13%-49%) (Figures 1 and 2), chronic pancreatitis-like parenchymal changes (14%-77%) (Figure 3) and solid lesions (0%-46%) (Figure 4), which are always considered suspicious[4,10,18,23,26,27,39,40]. Of note, most studies that reported a low rate of EUS-detected abnormalities did not consider chronic pancreatitis-like parenchymal changes.

Pancreatic cystic lesions identified in HRI are mainly IPMN, which are more frequent in HRI (13%-20%) than in the general population (1%-5%)[18,26,41,42]. Whether IPMN in HRI have a different course than sporadic ones is unknown, and hence their monitoring could be similar by analogy with the general population. First, the initial characterization of IPMN should search for potential high-risk stigmata (obstructive jaundice associated with cystic lesions of the head of the pancreas, enhancing mural nodules > 5 mm, main pancreatic duct > 10 mm) and worrisome features (pancreatitis, cyst > 3 cm, enhancing mural nodule < 5 mm, thickened/enhancing cyst walls, main duct size 5-9 mm, abrupt change in calibre of pancreatic duct with distal pancreatic atrophy, lymphadenopathy, increased CA19-9 serum levels, and cyst growth rate > 5 mm per 2 years)[43,44]. These features are highly important for deciding on surgery because their presence is associated with an increased probability of finding invasive PC or HGD on the resected specimen[11]. EUS has been shown to be the best technique for the early detection of malignancy in patients with IPMN[45]. Nevertheless, the entire pancreatic parenchyma is at risk of PC, which can occur away from the cysts[46]. Thus, surveillance should not focus on pancreatic cysts alone. However, all cystic lesions found in HRI are not IPMN (but may be other benign cysts such as serous cystadenomas)[15,30,31]. Nevertheless, in the absence of specific worrisome criteria for non-IPMN cystic pancreatic lesions, it seems appropriate to use the abovementioned worrisome features for all cystic lesions, while it may lead to operating on benign

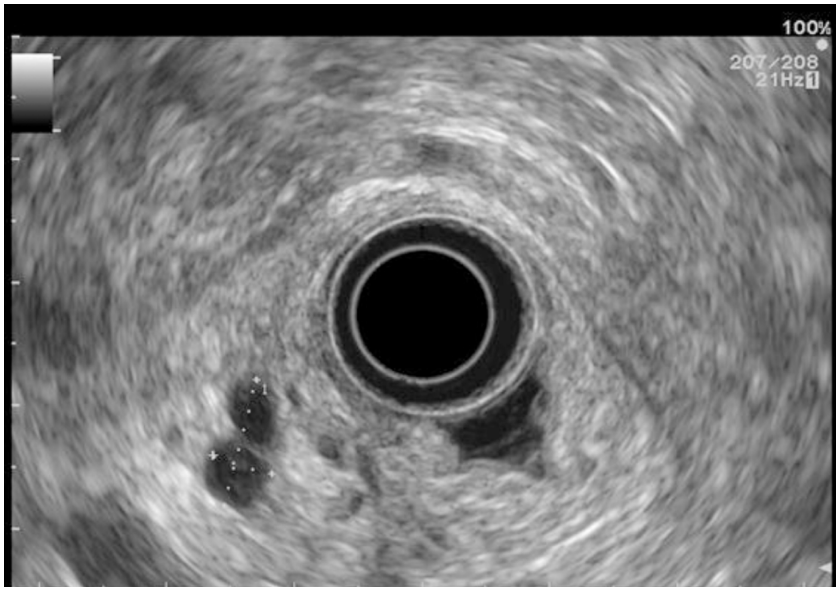

Figure 1 Pancreatic cystic lesion: Intraductal papillary mucinous neoplasm.

cysts without potential pejorative evolution[11].

Chronic-pancreatitis-like parenchymal changes (CPis) are more common in HRI (up to 67%-80%) than in the general population (15%-17%)[12,23,47-49]. The diagnostic criteria for chronic pancreatitis with EUS (Rosemont Criteria) include major criteria (hyperechoic foci with shadowing and main pancreatic duct calcification, a lobularity with honeycombing) and minor criteria (small cysts, dilated ducts ≥ 3.5 mm, irregular pancreatic ducts, dilated side branches ≥ 1 mm, hyperechoic duct walls, strands, non-shadowing hyperechoic foci, and lobularity with noncontiguous lobules)[50]. Chronic-pancreatitis-like parenchymal atrophy and hyperechoic foci may correspond to multifocal PanIN[51-54]. Brune *et al*[51] reported a significant correlation between CPis on EUS and percentage of PanIN lesions on surgical specimens. The supposed explanation is that multifocal PanIN lesions produce obstructive lobular atrophy, which is probably the source of CPis[53]. Features of chronic pancreatitis during EUS-based surveillance of HRI are easily seen with good interobserver agreement[48]. One study showed that CPis generally have no or little progression over time, although the follow-up period was limited (3 years)[48]. A fatty pancreas has also been reported to be a risk factor for PC and should be noted on the EUS report[55].

Finally, EUS can identify solid pancreatic lesions in up to 20% of HRI during follow-up. These solid tumours are generally PC but may also be PanIN with HGD or neuroendocrine tumours[10,11,15,26,31,32,40]. In published studies, 14/53 (26%) of operated significant lesions (for which MRI and EUS data were available) were only seen on EUS (Table 1) [12,15,23,27,31]. However, as previously reported, 7/26 HRI operated on for a "pancreatic mass" (27%) had lesions with no/low malignant potential on the pathological specimen[11]. Nevertheless, it is difficult to consider not operating on an HRI with a "pancreatic mass" identified during EUS screening[11]. As discussed below, lesion sampling may be of special interest in this setting.

Endoscopic ultrasound pancreatic screening techniques in high-risk individuals

A study by Shin *et al*[29] suggested that compared to radial EUS, linear-array EUS improves the detection of pancreatic lesions in HRI, probably due to better visualization of the pancreatic tail. This same study reported a "second-pass effect" with additional lesions detected during a second EUS examination[29].

The use of contrast-enhanced harmonic EUS (CH-EUS) could improve its diagnostic performance. Indeed, it can identify solid neoplastic components in pancreatic cysts/IPMN as hyperenhanced lesions (Figure 5)[56]. The pooled sensitivity and specificity of CH-EUS for the diagnosis of PC are very high (91% and 87%, respectively) in the general population with solid pancreatic neoplasms[56,57]. Nevertheless, there is no specific study on the use of CH-EUS for pancreatic screening in HRI.

EUS elastography could also be useful for the characterization of solid pancreatic lesions in this population[58]. Iglesias-Garcia *et al*[59] reported a sensitivity, a specificity, and positive and negative predictive values of 100%, 85.5%, 90.7%, and 100%, respectively, for the diagnosis of sporadic PC.

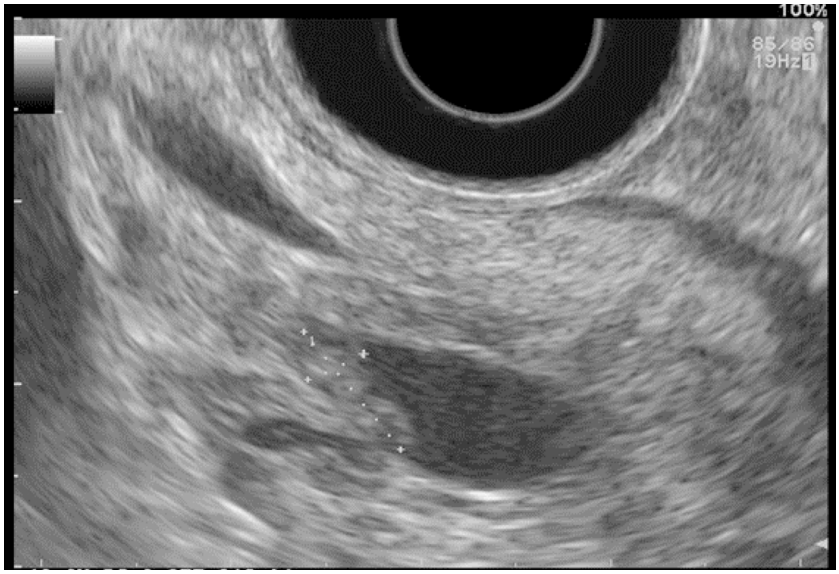

Figure 2 Intraductal papillary mucinous neoplasm with mural nodule.

The results of certain new techniques for the detection of (pre)malignant pancreatic lesions are especially promising in HRI, including the molecular imaging of cathepsin E *in vivo* using a confocal laser endomicroscopy miniprobe (overexpression of cathepsin E in PanIN) and the detection of DNA abnormalities or protein markers in pancreatic cyst fluid collected by fine needle aspiration (FNA) (mutations in genes such as *TP53*) (Figure 6)[57,60-63]. The role of these combined strategies (EUS with other new technological/biological techniques) as well as their impact on survival and cost-effectiveness must, however, still be defined[64]. Finally, another lead could also be the identification of molecular abnormalities (p53 mutations, *KRAS* mutations, DNA methylation markers) associated with the presence of PC and precursors of PC within pancreatic fluid collected by endoscopic retrograde cholangiopancreatography[65-69]. The role of these exams in FPC screening strategies must still be defined[68].

Endoscopic ultrasound-guided fine needle aspiration and fine needle biopsy

Another advantage of EUS is that FNA or fine needle biopsy (FNB) can be performed in solid pancreatic masses to obtain tissue samples for histopathological characterization with a low risk of complications[11,14,23,33,70]. The complication rate of EUS-FNA is approximately 2%[70]. The sensitivity (84.3%), specificity (97%), and accuracy (84%) of EUS-FNA for solid pancreatic masses are high[71]. However, the negative predictive value is low (64%)[26,71], indicating that a negative FNA does not exclude the presence of a (pre)malignant lesion. Thus, in the presence of a solid pancreatic lesion with negative histological samples, FNA (or FNB) must be repeated in HRI, and prophylactic pancreatectomy should be discussed. The use of cutting needles for EUS-FNB should improve pancreatic histological samples[72]. In addition, EUS-FNA is associated with a non-negligible rate of false positives (2% in the literature, up to 33% in HRI, especially in patients with CPis). Thus, the diagnostic value of FNA may be limited in HRI screening[11].

IMPROVING THE DETECTION OF HIGH-GRADE PANIN IN HIGH-RISK INDIVIDUALS

The theoretical risk of hereditary transmission of PC-predisposing genetic abnormalities is up to 50%. Hence, in the setting of FPC without a known predisposing gene mutation, approximately 50% of HRI undergo screening theoretically without predisposing genetic abnormalities. Thus, the detection of any cystic lesions or CPis is important in FPC-related HRI without identified mutations because it probably indicates that this subject carries the unidentified mutation and is hence at risk of PC.

Most PC arise from PanIN lesions, with a delay of approximately ten years[22]. The challenge of screening is being able to propose surgery at the stage of high-grade PanINs. Some aspects of high-grade PanINs have been described with MRI and EUS, but these lesions lack specificity[23,51,73]. Our group and others previously reported that

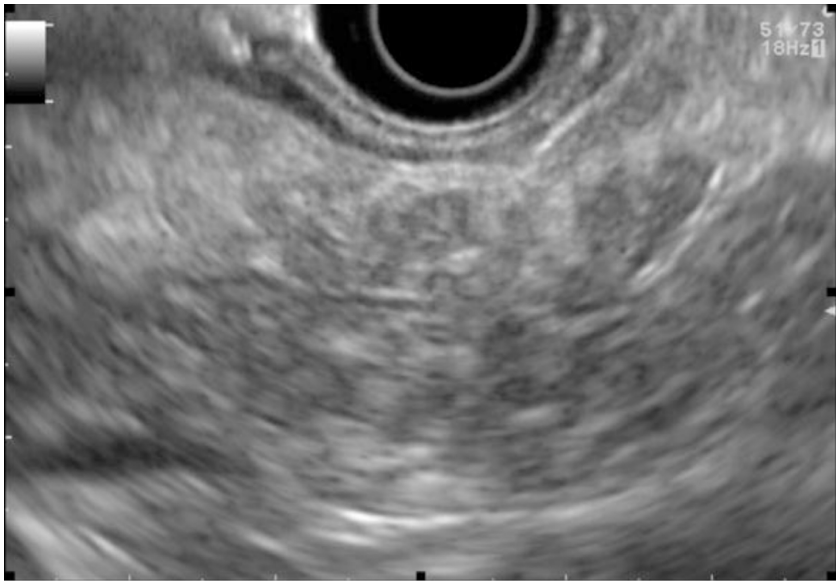

Figure 3 Chronic-pancreatitis-like parenchymal changes.

CPis (microcysts and/or hyperechoic foci of fibrosis) visualized at EUS were associated with PanIN (in up to 83% of cases)[51,54,74]. In an American study, the odds ratio for the association between intermediate-grade PanIN and hyperechoic foci without shadowing in the pancreas head was 8.5 (P = 0.05)[74]. This aspect would concern approximately 70% of HRI[12,23]. However, the assessment of such abnormalities might suffer from a lack of recognition, and the correlation between EUS aspect and pathology should be assessed in a large series of HRI[28,60].

Several studies have reported an increased risk of PC in patients with pancreatic cysts, whatever the etiology[73-75]. In some cases, and especially in HRI, MRI with MRCP and diffusion-weighted MR sequences can help identify very small cysts that do not communicate with the pancreatic ducts[73,74]. In a recent MRI study that included 100 patients, our group recently showed that the identification of non-communicating pancreatic microcysts had a 52.3% sensitivity, a 77.1% specificity, and a 61% accuracy for the diagnosis of PanIN[73]. In the same study, the association of global atrophy and non-communicating microcysts increased the predictive risk of PanIN. Interobserver agreement for the presence of microcysts was excellent with MRI (kappa = 0.92)[73].

PSYCHOLOGICAL IMPACT OF PANCREATIC SCREENING

The psychological burden in HRI of PC is significant because of the patient's level of risk and his/her experience with close relatives suffering from this severe disease[15]. Patients must be informed that the aim of screening is to identify premalignant lesions or early invasive PC to propose a pancreatectomy with prophylactic and/or curative intent. Additionally, they must be informed of the potential benefits (avoiding the development of PC, being treated at a curable stage, or at least diagnosing PC at the earliest stage possible and prolonging survival) and risks (related to general anaesthesia and/or EUS and especially FNA, unnecessary pancreatic surgery with a risk of complications and diabetes) of pancreatic screening[17]. Moreover, because the sensitivity of the different imaging examinations is not 100%, patients must be informed of the risk of missing (pre)malignant lesions and developing advanced PC during follow-up intervals.

Several studies have investigated the psychological burden of pancreatic screening and repeated examinations. Overall, pancreatic screening may have a positive psychological impact on HRI[76]. Konings *et al*[48] reported a low psychological burden due to the examinations themselves, which were considered uncomfortable only in 10% of cases. Of note, although it is an invasive procedure, EUS was generally not considered to be more uncomfortable than MRI[48,76].

COST-EFFECTIVENESS OF PANCREATIC SCREENING IN

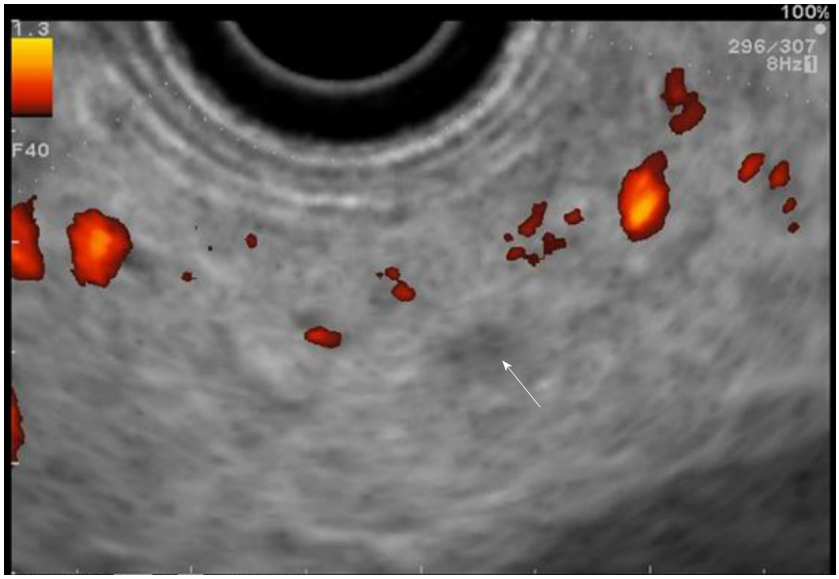

Figure 4 Small hypoechoic nodule.

HIGH-RISK INDIVIDUALS

It is difficult to assess the cost-effectiveness of pancreatic screening in HRI because of the different screening practices in different countries and the results of screening in different studies[10,11,17,18,23,24,40]. A screening test can generally be considered acceptable if there is a positive benefit/cost ratio. Rulyak *et al*[38] compared one-time EUS-based screening to no screening in a hypothetical cohort of 100 HRI. They showed that screening increased life expectancy (38 years) in a cost-effective manner ($ 16885 per life-year saved). Latchford *et al*[77] created a model that showed that seven PCs would theoretically be detected in 250 patients who would undergo yearly EUS between the age of 40 and 55 years old. The cost would be $ 164.285 dollars per PC detected and $ 372708 per life saved. Overall, it is not clear whether EUS-based screening can be considered cost-effective[60].

CONCLUSION

PC is inherited in 5%-10% of cases. HRI of PC can benefit from pancreatic screening mainly based on annual MRI and EUS. Successful screening targets are early invasive PC and IPMN or PanIN with HGD, which may be treated surgically with curative intent. These lesions are identified in 2% to 5% of all HRI undergoing screening. EUS appears to be the best examination to identify small solid lesions and is complementary to MRI, whose performance may be higher for identifying small cystic lesions. Pancreatic abnormalities found by EUS are cystic lesions (13%-49% of HRI), solid lesions (0%-46%) and chronic pancreatitis-like parenchymal changes (> 50%). Of note, the latter frequently correspond to PanIN lesions. Finally, CH-EUS, EUS-elastography and other new techniques (including needle-based confocal laser endomicroscopy miniprobe and the detection of DNA abnormalities or protein markers by FNA) are being developed, but further studies are needed to evaluate their role in the management of HRI. There is still limited evidence on the accuracy, acceptability and cost of screening as it is currently recommended. HRI undergoing screening should continue to be included in large cohorts, such as the CAPS consortium (http://caps-registry.com), which is a major opportunity to improve PC screening.

Areas of currently unmet needs within the scope of hereditary PC include the following: (1) To constitute large cohorts of HRI undergoing long-term prospective follow-up; (2) To study the correlation between the pancreatic abnormalities identified at imaging (MRI and EUS) and the lesions identified at the pathological examination of surgically resected specimens; (3) To develop specific EUS and MRI training modules to improve the recognition of pancreatic lesions in HRI and interobserver agreement; (4) To develop new techniques such as biomarkers or new nuclear imaging techniques; and (5) To study more comprehensively the consequences of screening in terms of cost-effectiveness, psychological burden, and

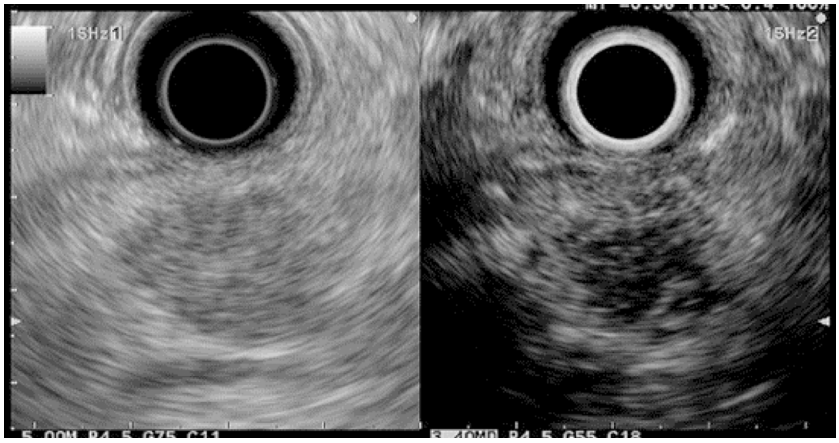

Figure 5 Contrast-enhanced harmonic in endoscopic ultrasound: Hypoechoic suspect nodule.

long-term surgical consequences in operated HRI.

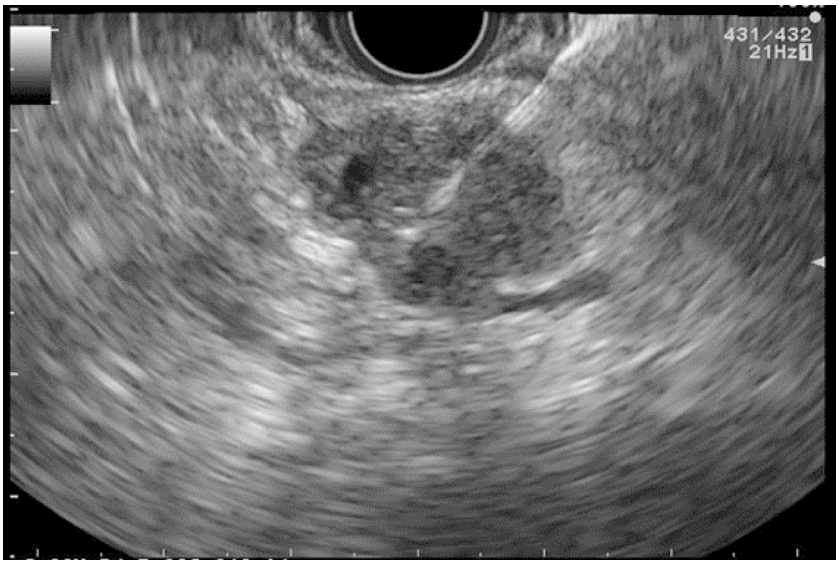

Figure 6 Fine needle aspiration of a solid pancreatic lesion.

REFERENCES

1 **Rahib L**, Smith BD, Aizenberg R, Rosenzweig AB, Fleshman JM, Matrisian LM. Projecting cancer incidence and deaths to 2030: the unexpected burden of thyroid, liver, and pancreas cancers in the United States. *Cancer Res* 2014; **74**: 2913-2921 [PMID: 24840647 DOI: 10.1158/0008-5472.CAN-14-0155]

2 **Neuzillet C**, Tijeras-Raballand A, Bourget P, Cros J, Couvelard A, Sauvanet A, Vullierme MP, Tournigand C, Hammel P. State of the art and future directions of pancreatic ductal adenocarcinoma therapy. *Pharmacol Ther* 2015; **155**: 80-104 [PMID: 26299994 DOI: 10.1016/j.pharmthera.2015.08.006]

3 **Harinck F**, Poley JW, Kluijt I, Fockens P, Bruno MJ; Dutch Research Group of Pancreatic Cancer Surveillance in High-Risk Individuals. Is early diagnosis of pancreatic cancer fiction? Surveillance of individuals at high risk for pancreatic cancer. *Dig Dis* 2010; **28**: 670-678 [PMID: 21088419 DOI: 10.1159/000320095]

4 **Bartsch DK**, Slater EP, Carrato A, Ibrahim IS, Guillen-Ponce C, Vasen HF, Matthäi E, Earl J, Jendryschek FS, Figiel J, Steinkamp M, Ramaswamy A, Vázquez-Sequeiros E, Muñoz-Beltran M, Montans J, Mocci E, Bonsing BA, Wasser M, Klöppel G, Langer P, Fendrich V, Gress TM. Refinement of screening for familial pancreatic cancer. *Gut* 2016; **65**: 1314-1321 [PMID: 27222532 DOI: 10.1136/gutjnl-2015-311098]

5 **Stoffel EM**. Screening in GI Cancers: The Role of Genetics. *J Clin Oncol* 2015; **33**: 1721-1728 [PMID: 25918283 DOI: 10.1200/JCO.2014.60.6764]

6 **Canto MI**, Harinck F, Hruban RH, Offerhaus GJ, Poley JW, Kamel I, Nio Y, Schulick RS, Bassi C, Kluijt I, Levy MJ, Chak A, Fockens P, Goggins M, Bruno M; International Cancer of Pancreas Screening (CAPS) Consortium. International Cancer of the Pancreas Screening (CAPS) Consortium summit on the management of patients with increased risk for familial pancreatic cancer. *Gut* 2013; **62**: 339-347 [PMID: 23135763 DOI: 10.1136/gutjnl-2012-303108]

7 **Kastrinos F**, Mukherjee B, Tayob N, Wang F, Sparr J, Raymond VM, Bandipalliam P, Stoffel EM, Gruber SB, Syngal S. Risk of pancreatic cancer in families with Lynch syndrome. *JAMA* 2009; **302**: 1790-1795 [PMID: 19861671 DOI: 10.1001/jama.2009.1529]

8 **Giardiello FM**, Brensinger JD, Tersmette AC, Goodman SN, Petersen GM, Booker SV, Cruz-Correa M, Offerhaus JA. Very high risk of cancer in familial Peutz-Jeghers syndrome. *Gastroenterology* 2000; **119**: 1447-1453 [PMID: 11113065 DOI: 10.1053/gast.2000.20228]

9 **Hu C**, Hart SN, Polley EC, Gnanaolivu R, Shimelis H, Lee KY, Lilyquist J, Na J, Moore R, Antwi SO, Bamlet WR, Chaffee KG, DiCarlo J, Wu Z, Samara R, Kasi PM, McWilliams RR, Petersen GM, Couch FJ. Association Between Inherited Germline Mutations in Cancer Predisposition Genes and Risk of Pancreatic Cancer. *JAMA* 2018; **319**: 2401-2409 [PMID: 29922827 DOI: 10.1001/jama.2018.6228]

10 **Mocci E**, Guillen-Ponce C, Earl J, Marquez M, Solera J, Salazar-López MT, Calcedo-Arnáiz C, Vázquez-Sequeiros E, Montans J, Muñoz-Beltrán M, Vicente-Bártulos A, González-Gordaliza C, Sanjuanbenito A, Guerrero C, Mendía E, Lisa E, Lobo E, Martínez JC, Real FX, Malats N, Carrato A. PanGen-Fam: Spanish registry of hereditary pancreatic cancer. *Eur J Cancer* 2015; **51**: 1911-1917 [PMID: 26212471 DOI: 10.1016/j.ejca.2015.07.004]

11 **de Mestier L**, Muller M, Cros J, Vullierme M-P, Vernerey D, Maire F, Dokmak S, Rebours V, Sauvanet A, Lévy P, Hammel P. Appropriateness of pancreatic resection in high-risk individuals for familial pancreatic ductal adenocarcinoma: a patient-level meta-analysis and proposition of the Beaujon score. *United Eur Gastroenterol J* 2019; **7**: 358-368 [PMID: 31019704 DOI: 10.1177/2050640618824910]

12 **Canto MI**, Goggins M, Yeo CJ, Griffin C, Axilbund JE, Brune K, Ali SZ, Jagannath S, Petersen GM, Fishman EK, Piantadosi S, Giardiello FM, Hruban RH. Screening for pancreatic neoplasia in high-risk individuals: an EUS-based approach. *Clin Gastroenterol Hepatol* 2004; **2**: 606-621 [PMID: 15224285 DOI: 10.1016/S1542-3565(04)00244-7]

13 **Poley JW**, Kluijt I, Gouma DJ, Harinck F, Wagner A, Aalfs C, van Eijck CH, Cats A, Kuipers EJ, Nio Y, Fockens P, Bruno MJ. The yield of first-time endoscopic ultrasonography in screening individuals at a high risk of developing pancreatic cancer. *Am J Gastroenterol* 2009; **104**: 2175-2181 [PMID: 19491823 DOI: 10.1038/ajg.2009.276]

14 **Sud A**, Wham D, Catalano M, Guda NM. Promising outcomes of screening for pancreatic cancer by genetic testing and endoscopic ultrasound. *Pancreas* 2014; **43**: 458-461 [PMID: 24622079 DOI: 10.1097/MPA.0000000000000052]

15 **Langer P**, Kann PH, Fendrich V, Habbe N, Schneider M, Sina M, Slater EP, Heverhagen JT, Gress TM, Rothmund M, Bartsch DK. Five years of prospective screening of high-risk individuals from families with familial pancreatic cancer. *Gut* 2009; **58**: 1410-1418 [PMID: 19470496 DOI: 10.1136/gut.2008.171611]

16 **Zubarik R**, Gordon SR, Lidofsky SD, Anderson SR, Pipas JM, Badger G, Ganguly E, Vecchio J. Screening for pancreatic cancer in a high-risk population with serum CA 19-9 and targeted EUS: a feasibility study. *Gastrointest Endosc* 2011; **74**: 87-95 [PMID: 21704809 DOI: 10.1016/j.gie.2011.03.1235]

17 **Stoffel EM**, McKernin SE, Khorana AA. Evaluating Susceptibility to Pancreatic Cancer: ASCO Clinical Practice Provisional Clinical Opinion Summary. *J Oncol Pract* 2019; **15**: 108-111 [PMID: 30589608 DOI: 10.1200/JOP.18.00629]

18 **Signoretti M**, Bruno MJ, Zerboni G, Poley JW, Delle Fave G, Capurso G. Results of surveillance in individuals at high-risk of pancreatic cancer: A systematic review and meta-analysis. *United European Gastroenterol J* 2018; **6**: 489-499 [PMID: 29881603 DOI: 10.1177/2050640617752182]

19 **Corral JE**, Mareth KF, Riegert-Johnson DL, Das A, Wallace MB. Diagnostic Yield From Screening Asymptomatic Individuals at High Risk for Pancreatic Cancer: A Meta-analysis of Cohort Studies. *Clin Gastroenterol Hepatol* 2019; **17**: 41-53 [PMID: 29775792 DOI: 10.1016/j.cgh.2018.04.065]

20 **Das KK**, Early D. Pancreatic Cancer Screening. *Curr Treat Options Gastroenterol* 2017; **15**: 562-575 [PMID: 28879469 DOI: 10.1007/s11938-017-0149-8]

21 **Rebours V**, Lévy P, Mosnier JF, Scoazec JY, Soubeyrand MS, Fléjou JF, Turlin B, Hammel P, Ruszniewski P, Bedossa P, Couvelard A. Pathology analysis reveals that dysplastic pancreatic ductal lesions are frequent in patients with hereditary pancreatitis. *Clin Gastroenterol Hepatol* 2010; **8**: 206-212 [PMID: 19765677 DOI: 10.1016/j.cgh.2009.09.009]

22 **Yachida S**, Jones S, Bozic I, Antal T, Leary R, Fu B, Kamiyama M, Hruban RH, Eshleman JR, Nowak MA, Velculescu VE, Kinzler KW, Vogelstein B, Iacobuzio-Donahue CA. Distant metastasis occurs late during the genetic evolution of pancreatic cancer. *Nature* 2010; **467**: 1114-1117 [PMID: 20981102 DOI: 10.1038/nature09515]

23 **Canto MI**, Goggins M, Hruban RH, Petersen GM, Giardiello FM, Yeo C, Fishman EK, Brune K, Axilbund J, Griffin C, Ali S, Richman J, Jagannath S, Kantsevoy SV, Kalloo AN. Screening for early pancreatic neoplasia in high-risk individuals: a prospective controlled study. *Clin Gastroenterol Hepatol* 2006; **4**: 766-81; quiz 665 [PMID: 16682259 DOI: 10.1016/j.cgh.2006.02.005]

24 **Yasuda I**, Iwashita T, Doi S, Nakashima M, Moriwaki H. Role of EUS in the early detection of small pancreatic cancer. *Dig Endosc* 2011; **23** Suppl 1: S22-25 [PMID: 21535195 DOI: 10.1111/j.1443-1661.2011.01113.x]

25 **Canto MI**, Hruban RH, Fishman EK, Kamel IR, Schulick R, Zhang Z, Topazian M, Takahashi N, Fletcher J, Petersen G, Klein AP, Axilbund J, Griffin C, Syngal S, Saltzman JR, Mortele KJ, Lee J, Tamm E, Vikram R, Bhosale P, Margolis D, Farrell J, Goggins M; American Cancer of the Pancreas Screening (CAPS) Consortium. Frequent detection of pancreatic lesions in asymptomatic high-risk individuals. *Gastroenterology* 2012; **142**: 796-804 [PMID: 22245846 DOI: 10.1053/j.gastro.2012.01.005]

26 **Capurso G**, Signoretti M, Valente R, Arnelo U, Lohr M, Poley JW, Delle Fave G, Del Chiaro M. Methods and outcomes of screening for pancreatic adenocarcinoma in high-risk individuals. *World J Gastrointest Endosc* 2015; **7**: 833-842 [PMID: 26240684 DOI: 10.4253/wjge.v7.i9.833]

27 **Harinck F**, Konings IC, Kluijt I, Poley JW, van Hooft JE, van Dullemen HM, Nio CY, Krak NC, Hermans JJ, Aalfs CM, Wagner A, Sijmons RH, Biermann K, van Eijck CH, Gouma DJ, Dijkgraaf MG, Fockens P, Bruno MJ; Dutch research group on pancreatic cancer surveillance in high-risk individuals. A multicentre comparative prospective blinded analysis of EUS and MRI for screening of pancreatic cancer in high-risk individuals. *Gut* 2016; **65**: 1505-1513 [PMID: 25986944 DOI: 10.1136/gutjnl-2014-308008]

28 **Topazian M**, Enders F, Kimmey M, Brand R, Chak A, Clain J, Cunningham J, Eloubeidi M, Gerdes H, Gress F, Jagannath S, Kantsevoy S, LeBlanc JK, Levy M, Lightdale C, Romagnuolo J, Saltzman JR, Savides T, Wiersema M, Woodward T, Petersen G, Canto M. Interobserver agreement for EUS findings in familial pancreatic-cancer kindreds. *Gastrointest Endosc* 2007; **66**: 62-67 [PMID: 17382940 DOI: 10.1016/j.gie.2006.09.018]

29 **Shin EJ**, Topazian M, Goggins MG, Syngal S, Saltzman JR, Lee JH, Farrell JJ, Canto MI. Linear-array EUS improves detection of pancreatic lesions in high-risk individuals: a randomized tandem study. *Gastrointest Endosc* 2015; **82**: 812-818 [PMID: 25930097 DOI: 10.1016/j.gie.2015.02.028]

30 **Canto MI**, Almario JA, Schulick RD, Yeo CJ, Klein A, Blackford A, Shin EJ, Sanyal A, Yenokyan G, Lennon AM, Kamel IR, Fishman EK, Wolfgang C, Weiss M, Hruban RH, Goggins M. Risk of Neoplastic Progression in Individuals at High Risk for Pancreatic Cancer Undergoing Long-term Surveillance. *Gastroenterology* 2018; **155**: 740-751 [PMID: 29803839 DOI: 10.1053/j.gastro.2018.05.035]

31 **Vasen H**, Ibrahim I, Ponce CG, Slater EP, Matthäi E, Carrato A, Earl J, Robbers K, van Mil AM, Potjer T, Bonsing BA, de Vos Tot Nederveen Cappel WH, Bergman W, Wasser M, Morreau H, Klöppel G, Schicker C, Steinkamp M, Figiel J, Esposito I, Mocci E, Vazquez-Sequeiros E, Sanjuanbenito A, Muñoz-Beltran M, Montans J, Langer P, Fendrich V, Bartsch DK. Benefit of Surveillance for Pancreatic Cancer in High-Risk Individuals: Outcome of Long-Term Prospective Follow-Up Studies From Three European Expert Centers. *J Clin Oncol* 2016; **34**: 2010-2019 [PMID: 27114589 DOI: 10.1200/JCO.2015.64.0730]

32 **Al-Sukhni W**, Borgida A, Rothenmund H, Holter S, Semotiuk K, Grant R, Wilson S, Moore M, Narod S, Jhaveri K, Haider MA, Gallinger S. Screening for pancreatic cancer in a high-risk cohort: an eight-year experience. *J Gastrointest Surg* 2012; **16**: 771-783 [PMID: 22127781 DOI: 10.1007/s11605-011-1781-6]

33 **Verna EC**, Hwang C, Stevens PD, Rotterdam H, Stavropoulos SN, Sy CD, Prince MA, Chung WK, Fine RL, Chabot JA, Frucht H. Pancreatic cancer screening in a prospective cohort of high-risk patients: a comprehensive strategy of imaging and genetics. *Clin Cancer Res* 2010; **16**: 5028-5037 [PMID: 20876795 DOI: 10.1158/1078-0432.CCR-09-3209]

34 **Ludwig E**, Olson SH, Bayuga S, Simon J, Schattner MA, Gerdes H, Allen PJ, Jarnagin WR, Kurtz RC. Feasibility and yield of screening in relatives from familial pancreatic cancer families. *Am J Gastroenterol* 2011; **106**: 946-954 [PMID: 21468009 DOI: 10.1038/ajg.2011.65]

35 **Imbe K**, Nagata N, Hisada Y, Takasaki Y, Sekine K, Mishima S, Kawazoe A, Tajima T, Shimbo T, Yanase M, Akiyama J, Fujimoto K, Uemura N. Validation of the American Gastroenterological Association guidelines on management of intraductal papillary mucinous neoplasms: more than 5 years of follow-up. *Eur Radiol* 2018; **28**: 170-178 [PMID: 28770404 DOI: 10.1007/s00330-017-4966-x]

36 **Gangi A**, Malafa M, Klapman J. Endoscopic Ultrasound-Based Pancreatic Cancer Screening of High-Risk Individuals: A Prospective Observational Trial. *Pancreas* 2018; **47**: 586-591 [PMID: 29683970 DOI: 10.1097/MPA.0000000000001038]

37 **Brentnall TA**, Bronner MP, Byrd DR, Haggitt RC, Kimmey MB. Early diagnosis and treatment of pancreatic dysplasia in patients with a family history of pancreatic cancer. *Ann Intern Med* 1999; **131**: 247-255 [PMID: 10454945 DOI: 10.7326/0003-4819-131-4-199908170-00003]

38 **Rulyak SJ**, Kimmey MB, Veenstra DL, Brentnall TA. Cost-effectiveness of pancreatic cancer screening in familial pancreatic cancer kindreds. *Gastrointest Endosc* 2003; **57**: 23-29 [PMID: 12518126 DOI: 10.1067/mge.2003.28]

39 **DaVee T**, Coronel E, Papafragkakis C, Thaiudom S, Lanke G, Chakinala RC, Nogueras González GM, Bhutani MS, Ross WA, Weston BR, Lee JH. Pancreatic cancer screening in high-risk individuals with germline genetic mutations. *Gastrointest Endosc* 2018; **87**: 1443-1450 [PMID: 29309780 DOI: 10.1016/j.gie.2017.12.019]

40 **Schneider R**, Slater EP, Sina M, Habbe N, Fendrich V, Matthäi E, Langer P, Bartsch DK. German national case collection for familial pancreatic cancer (FaPaCa): ten years experience. *Fam Cancer* 2011; **10**: 323-330 [PMID: 21207249 DOI: 10.1007/s10689-010-9414-x]

41 **Roch AM**, Schneider J, Carr RA, Lancaster WP, House MG, Zyromski NJ, Nakeeb A, Schmidt CM, Ceppa EP. Are BRCA1 and BRCA2 gene mutation patients underscreened for pancreatic adenocarcinoma? *J Surg Oncol* 2019; **119**: 777-783 [PMID: 30636051 DOI: 10.1002/jso.25376]

42 **Zanini N**, Giordano M, Smerieri E, Cipolla d'Abruzzo G, Guidi M, Pazzaglini G, De Luca F, Chiaruzzi G, Vitullo G, Piva P, Lombardi R, Jovine E, Gatti M, Landolfo G. Estimation of the prevalence of asymptomatic pancreatic cysts in the population of San Marino. *Pancreatology* 2015; **15**: 417-422 [PMID: 26028332 DOI: 10.1016/j.pan.2015.05.461]

43 **Tanaka M**, Fernández-Del Castillo C, Kamisawa T, Jang JY, Levy P, Ohtsuka T, Salvia R, Shimizu Y, Tada M, Wolfgang CL. Revisions of international consensus Fukuoka guidelines for the management of IPMN of the pancreas. *Pancreatology* 2017; **17**: 738-753 [PMID: 28735806 DOI: 10.1016/j.pan.2017.07.007]

44 **European Study Group on Cystic Tumours of the Pancreas**. European evidence-based guidelines on pancreatic cystic neoplasms. *Gut* 2018; **67**: 789-804 [PMID: 29574408 DOI: 10.1136/gutjnl-2018-316027]

45 **Kamata K**, Kitano M, Kudo M, Sakamoto H, Kadosaka K, Miyata T, Imai H, Maekawa K, Chikugo T, Kumano M, Hyodo T, Murakami T, Chiba Y, Takeyama Y. Value of EUS in early detection of pancreatic ductal adenocarcinomas in patients with intraductal papillary mucinous neoplasms. *Endoscopy* 2014; **46**: 22-29 [PMID: 24218310 DOI: 10.1055/s-0033-1353603]

46 **Felsenstein M**, Noë M, Masica DL, Hosoda W, Chianchiano P, Fischer CG, Lionheart G, Brosens LAA, Pea A, Yu J, Gemenetzis G, Groot VP, Makary MA, He J, Weiss MJ, Cameron JL, Wolfgang CL, Hruban RH, Roberts NJ, Karchin R, Goggins MG, Wood LD. IPMNs with co-occurring invasive cancers: neighbours but not always relatives. *Gut* 2018; **67**: 1652-1662 [PMID: 29500184 DOI: 10.1136/gutjnl-2017-315062]

47 **Petrone MC**, Arcidiacono PG, Perri F, Carrara S, Boemo C, Testoni PA. Chronic pancreatitis-like changes detected by endoscopic ultrasound in subjects without signs of pancreatic disease: do these indicate age-related changes, effects of xenobiotics, or early chronic pancreatitis? *Pancreatology* 2010; **10**: 597-602 [PMID: 20980777 DOI: 10.1159/000314599]

48 **Konings ICAW**, Cahen DL, Harinck F, Fockens P, van Hooft JE, Poley JW, Bruno MJ. Evolution of features of chronic pancreatitis during endoscopic ultrasound-based surveillance of individuals at high risk for pancreatic cancer. *Endosc Int Open* 2018; **6**: E541-E548 [PMID: 29713680 DOI: 10.1055/a-0574-2396]

49 **Thiruvengadam SS**, Chuang J, Huang R, Girotra M, Park WG. Chronic pancreatitis changes in high-risk individuals for pancreatic ductal adenocarcinoma. *Gastrointest Endosc* 2019; **89**: 842-851 [PMID: 30145314 DOI: 10.1016/j.gie.2018.08.029]

50 **Catalano MF**, Sahai A, Levy M, Romagnuolo J, Wiersema M, Brugge W, Freeman M, Yamao K, Canto M, Hernandez LV. EUS-based criteria for the diagnosis of chronic pancreatitis: the Rosemont classification. *Gastrointest Endosc* 2009; **69**: 1251-1261 [PMID: 19243769 DOI: 10.1016/j.gie.2008.07.043]

51 **Brune K**, Abe T, Canto M, O'Malley L, Klein AP, Maitra A, Volkan Adsay N, Fishman EK, Cameron JL, Yeo CJ, Kern SE, Goggins M, Hruban RH. Multifocal neoplastic precursor lesions associated with lobular atrophy of the pancreas in patients having a strong family history of pancreatic cancer. *Am J Surg Pathol* 2006; **30**: 1067-1076 [PMID: 16931950 DOI: pas.0000213265.84725.0b]

52 **Hruban RH**, Canto MI, Goggins M, Schulick R, Klein AP. Update on familial pancreatic cancer. *Adv Surg* 2010; **44**: 293-311 [PMID: 20919528 DOI: 10.1016/j.yasu.2010.05.011]

53 **Aimoto T**, Uchida E, Nakamura Y, Matsushita A, Katsuno A, Chou K, Kawamoto M, Naito Z, Tajiri T. Multicentric pancreatic intraepithelial neoplasias (PanINs) presenting with the clinical features of chronic pancreatitis. *J Hepatobiliary Pancreat Surg* 2008; **15**: 549-553 [PMID: 18836812 DOI: 10.1007/s00534-007-1269-7]

54 **Maire F**, Couvelard A, Palazzo L, Aubert A, Vullierme MP, Rebours V, Hammel P, Sauvanet A, Levy P, Ruszniewski P. Pancreatic intraepithelial neoplasia in patients with intraductal papillary mucinous neoplasms: the interest of endoscopic ultrasonography. *Pancreas* 2013; **42**: 1262-1266 [PMID: 24152960 DOI: 10.1097/01.mpa.0000437639.38383.41]

55 **Lesmana CRA**, Gani RA, Lesmana LA. Non-alcoholic fatty pancreas disease as a risk factor for pancreatic cancer based on endoscopic ultrasound examination among pancreatic cancer patients: A single-center experience. *JGH Open* 2017; **2**: 4-7 [PMID: 30483555 DOI: 10.1002/jgh3.12032]

56 **Fusaroli P**, Napoleon B, Gincul R, Lefort C, Palazzo L, Palazzo M, Kitano M, Minaga K, Caletti G, Lisotti A. The clinical impact of ultrasound contrast agents in EUS: a systematic review according to the levels of evidence. *Gastrointest Endosc* 2016; **84**: 587-596 [PMID: 27311654 DOI: 10.1016/j.gie.2016.06.006]

57 **Li XZ**, Song J, Sun ZX, Yang YY, Wang H. Diagnostic performance of contrast-enhanced ultrasound for pancreatic neoplasms: A systematic review and meta-analysis. *Dig Liver Dis* 2018; **50**: 132-138 [PMID: 29162410 DOI: 10.1016/j.dld.2017.10.012]

58 **Kawada N**, Tanaka S. Elastography for the pancreas: Current status and future perspective. *World J Gastroenterol* 2016; **22**: 3712-3724 [PMID: 27076756 DOI: 10.3748/wjg.v22.i14.3712]

59 **Iglesias-Garcia J**, Larino-Noia J, Abdulkader I, Forteza J, Dominguez-Munoz JE. EUS elastography for the characterization of solid pancreatic masses. *Gastrointest Endosc* 2009; **70**: 1101-1108 [PMID:

19647248 DOI: 10.1016/j.gie.2009.05.011]
60 **Lami G**, Biagini MR, Galli A. Endoscopic ultrasonography for surveillance of individuals at high risk for pancreatic cancer. *World J Gastrointest Endosc* 2014; **6**: 272-285 [PMID: 25031786 DOI: 10.4253/wjge.v6.i7.272]
61 **Bhutani MS**, Koduru P, Joshi V, Saxena P, Suzuki R, Irisawa A, Yamao K. The role of endoscopic ultrasound in pancreatic cancer screening. *Endosc Ultrasound* 2016; **5**: 8-16 [PMID: 26879161 DOI: 10.4103/2303-9027.175876]
62 **Kanda M**, Sadakari Y, Borges M, Topazian M, Farrell J, Syngal S, Lee J, Kamel I, Lennon AM, Knight S, Fujiwara S, Hruban RH, Canto MI, Goggins M. Mutant TP53 in duodenal samples of pancreatic juice from patients with pancreatic cancer or high-grade dysplasia. *Clin Gastroenterol Hepatol* 2013; **11**: 719-30 [PMID: 23200980 DOI: 10.1016/j.cgh.2012.11.016]
63 **Bournet B**, Gayral M, Torrisani J, Selves J, Cordelier P, Buscail L. Role of endoscopic ultrasound in the molecular diagnosis of pancreatic cancer. *World J Gastroenterol* 2014; **20**: 10758-10768 [PMID: 25152579 DOI: 10.3748/wjg.v20.i31.10758]
64 **Bhutani MS**, Gupta V, Guha S, Gheonea DI, Saftoiu A. Pancreatic cyst fluid analysis--a review. *J Gastrointestin Liver Dis* 2011; **20**: 175-180 [PMID: 21725515]
65 **Wong T**, Howes N, Threadgold J, Smart HL, Lombard MG, Gilmore I, Sutton R, Greenhalf W, Ellis I, Neoptolemos JP. Molecular diagnosis of early pancreatic ductal adenocarcinoma in high-risk patients. *Pancreatology* 2001; **1**: 486-509 [PMID: 12120229 DOI: 10.1159/000055852]
66 **Kisiel JB**, Raimondo M, Taylor WR, Yab TC, Mahoney DW, Sun Z, Middha S, Baheti S, Zou H, Smyrk TC, Boardman LA, Petersen GM, Ahlquist DA. New DNA Methylation Markers for Pancreatic Cancer: Discovery, Tissue Validation, and Pilot Testing in Pancreatic Juice. *Clin Cancer Res* 2015; **21**: 4473-4481 [PMID: 26023084 DOI: 10.1158/1078-0432.CCR-14-2469]
67 **Takano S**, Fukasawa M, Kadokura M, Shindo H, Takahashi E, Hirose S, Maekawa S, Mochizuki K, Kawaida H, Itakura J, Katoh R, Fujii H, Sato T, Enomoto N. Next-Generation Sequencing Revealed TP53 Mutations to Be Malignant Marker for Intraductal Papillary Mucinous Neoplasms That Could Be Detected Using Pancreatic Juice. *Pancreas* 2017; **46**: 1281-1287 [PMID: 28930868 DOI: 10.1097/MPA.0000000000000931]
68 **Yan L**, McFaul C, Howes N, Leslie J, Lancaster G, Wong T, Threadgold J, Evans J, Gilmore I, Smart H, Lombard M, Neoptolemos J, Greenhalf W. Molecular analysis to detect pancreatic ductal adenocarcinoma in high-risk groups. *Gastroenterology* 2005; **128**: 2124-2130 [PMID: 15940643 DOI: 10.1053/j.gastro.2005.03.006]
69 **Matsumoto K**, Takeda Y, Harada K, Onoyama T, Kawata S, Horie Y, Sakamoto T, Ueki M, Miura N, Murawaki Y. Clinical Impact of the KL-6 Concentration of Pancreatic Juice for Diagnosing Pancreatic Masses. *Biomed Res Int* 2015; **2015**: 528304 [PMID: 26451373 DOI: 10.1155/2015/528304]
70 **Gonzalo-Marin J**, Vila JJ, Perez-Miranda M. Role of endoscopic ultrasound in the diagnosis of pancreatic cancer. *World J Gastrointest Oncol* 2014; **6**: 360-368 [PMID: 25232461 DOI: 10.4251/wjgo.v6.i9.360]
71 **Eloubeidi MA**, Chen VK, Eltoum IA, Jhala D, Chhieng DC, Jhala N, Vickers SM, Wilcox CM. Endoscopic ultrasound-guided fine needle aspiration biopsy of patients with suspected pancreatic cancer: diagnostic accuracy and acute and 30-day complications. *Am J Gastroenterol* 2003; **98**: 2663-2668 [PMID: 14687813 DOI: 10.1111/j.1572-0241.2003.08666.x]
72 **Tian L**, Tang AL, Zhang L, Liu XW, Li JB, Wang F, Shen SR, Wang XY. Evaluation of 22G fine-needle aspiration (FNA) versus fine-needle biopsy (FNB) for endoscopic ultrasound-guided sampling of pancreatic lesions: a prospective comparison study. *Surg Endosc* 2018; **32**: 3533-3539 [PMID: 29404729 DOI: 10.1007/s00464-018-6075-6]
73 **Vullierme MP**, Menassa L, Couvelard A, Rebours V, Maire F, Ibrahim T, Cros J, Ruszniewski P, Sauvanet A, Levy P, Soyer P, Vilgrain V. Non-branched microcysts of the pancreas on MR imaging of patients with pancreatic tumors who had pancreatectomy may predict the presence of pancreatic intraepithelial neoplasia (PanIN): a preliminary study. *Eur Radiol* 2019 [PMID: 30972547 DOI: 10.1007/s00330-019-06154-3]
74 **LeBlanc JK**, Chen JH, Al-Haddad M, Luz L, McHenry L, Sherman S, Juan M, Dewitt J. Can endoscopic ultrasound predict pancreatic intraepithelial neoplasia lesions in chronic pancreatitis?: a retrospective study of pathologic correlation. *Pancreas* 2014; **43**: 849-854 [PMID: 24826885 DOI: 10.1097/MPA.0000000000000142]
75 **Munigala S**, Gelrud A, Agarwal B. Risk of pancreatic cancer in patients with pancreatic cyst. *Gastrointest Endosc* 2016; **84**: 81-86 [PMID: 26524643 DOI: 10.1016/j.gie.2015.10.030]
76 **Cazacu IM**, Luzuriaga Chavez AA, Saftoiu A, Bhutani MS. Psychological impact of pancreatic cancer screening by EUS or magnetic resonance imaging in high-risk individuals: A systematic review. *Endosc Ultrasound* 2019; **8**: 17-24 [PMID: 30246710 DOI: 10.4103/eus.eus_25_18]
77 **Latchford A**, Greenhalf W, Vitone LJ, Neoptolemos JP, Lancaster GA, Phillips RK. Peutz-Jeghers syndrome and screening for pancreatic cancer. *Br J Surg* 2006; **93**: 1446-1455 [PMID: 17115408 DOI: 10.1002/bjs.5609]
78 **Kimmey MB**, Bronner MP, Byrd DR, Brentnall TA. Screening and surveillance for hereditary pancreatic cancer. *Gastrointest Endosc* 2002; **56**: S82-S86 [PMID: 12297755 DOI: 10.1016/s0016-5107(02)70092-8]

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MINIREVIEWS

Optimizing proton pump inhibitors in *Helicobacter pylori* treatment: Old and new tricks to improve effectiveness

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Abstract

The survival and replication cycle of *Helicobacter pylori* (*H. pylori*) is strictly dependant on intragastric pH, since *H. pylori* enters replicative phase at an almost neutral pH (6-7), while at acid pH (3-6) it turns into its coccoid form, which is resistant to antibiotics. On these bases, it is crucial to increase intragastric pH by proton pump inhibitors (PPIs) when an antibiotic-based eradicating therapy needs to be administered. Therefore, several tricks need to be used to optimize eradication rate of different regimens. The administration of the highest dose as possible of PPI, by doubling or increasing the number of pills/day, has shown to be able to improve therapeutic outcome and has often proposed in rescue therapies, even if specific trials have not been performed. A pre-treatment with PPI before starting antibiotics does not seem to be effective, therefore it is discouraged. However, the choice of PPI molecule could have a certain weight, since second-generation substances (esomeprazole, rabeprazole) are likely more effective than those of first generation (omeprazole, lansoprazole). A possible explanation is due to their metabolism, which has been proven to be less dependent on cytochrome P450 (CYP) 2C19 genetic variables. Finally, vonoprazan, a competitive inhibitor of H+/K+-ATPase present on luminal membrane of gastric parietal cells has shown the highest efficacy, due to both its highest acid inhibition power and rapid pharmacologic effect. However current data come only from Eastern Asia, therefore its strong power needs to be confirmed outside this geographic area in Western countries as well as related to the local different antibiotic resistance rates.

Key words: *Helicobacter pylori*; Proton pump inhibitors; Eradication; Cytochrome P450; Optimization

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Core tip: The most adequate acid suppression by proton pump inhibitors (PPIs) is fundamental to optimize antibiotic therapies for *Helicobacter pylori* eradication. Herein, we summarize data from literature in order to ascertain the most effective strategies in this topic. Increasing PPI dose showed a real benefit on eradication rate even in the absence of dedicated trials. Second-generation PPIs, as demonstrated in some meta-analyses, may be more effective than old PPI molecules. Finally, vonoprazan, a novel molecule, has shown promising results but, currently, data come from Asian countries, therefore its strong power needs to be confirmed outside this geographic area.

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INTRODUCTION

Helicobacter pylori (*H. pylori*) is a microaerofilic Gram-negative bacterium that colonizes the human stomach thus causing gastritis, peptic ulcer and malignant diseases (gastric cancer and mucosa associated lymphoid tissue lymphoma)[1]. Therefore, a prompt eradication of *H. pylori* may heal non-malignant conditions and stop the development of neoplastic diseases.

Until few decades ago, the human stomach was considered as a "sterile" organ where the acidic pH hampered the growth of any germ. However, the discovery of *H. pylori* has disavowed this axiom[2]. Urease is the most important enzyme of this bacterium, since it allows *H. pylori* stomach colonization by degrading urea into carbon dioxide and ammonia, an alkaline molecule able to counteract acidic environment below the layer of neutral mucins that cover the gastric wall[3]. Thus, the survival and growth of *H. pylori* is strictly dependant on urease effect on intragastric pH at this site. Indeed, it has been observed that only bacteria in replicative vegetative phase are susceptible to antibiotics[4-7]. *H. pylori* enters replicative phase at an almost neutral pH (6-7), while at acid pH (3-6) it turns into the coccoid form that is resistant to antibiotics[4-9]. On these bases, it is crucial to increase intragastric pH by proton pump inhibitors (PPIs) when an antibiotic-based eradicating therapy needs to be administered, since an inadequate acid suppression may keep some bacteria in non replicative forms, not susceptible to antibiotics. On the other hand, this mechanism explains some cases of treatment failure, not linked to bacterial genotypic resistance[8]. A further relevant property of PPIs is the ability to reduce intragastric bacterial load, thus making more likely the success of antibiotics. This relevant aspect is supported by the observation that patients with very high bacterial load are the most resistant ones to therapeutic approaches[10,11]. Finally, an additional demonstration in favor of PPI role in *H. pylori* treatment was suggested by Figura *et al*[12], who showed that lansoprazole reduces minimal inhibitory concentration of cultured *H. pylori* strains through a possible direct inhibitory effect on bacterial replication. The evidence of the pivotal usefulness of a correct use of PPIs in the eradication of *H. pylori* led us to perform a narrative review aimed to explore some poorly considered aspects of PPI management in this context.

THE OPTIMAL DOSE

Current Maastricht V guidelines[13] recommend high PPI dose, which is equivalent to omeprazole 20 mg, lansoprazole 30 mg, pantoprazole 40 mg or rabeprazole 20 mg, all twice daily, while low doses are discouraged since they are ineffective, as clearly highlighted in 2015 Italian guidelines[14]. Therefore, a very high dose is defined by both doubling usual amount (*e.g.*, lansoprazole 60 mg bid) or multiple administration (*e.g.*, lansoprazole 30 mg tid or qid). At this regard, there is no unanimous consent for the definition of the very high dose of esomeprazole, since some Authors suggest 20 mg bid and others 40 mg bid[15,16]. In the OPTRICON trial[15], esomeprazole 40 mg bid was given with a 14-d triple therapy in 402 naïve patients, with an eradication rate at intention to treat of 81.3%. Unfortunately, in this trial the alternative arm was

represented by concomitant therapy, therefore it was inadequate to give useful information about the PPI dose effectiveness. On the other hand, De Francesco *et al*[16] showed that esomeprazole 40 mg bid with 14-d triple therapy showed eradication rate of 81.9% compared to 73.9% of esomeprazole 20 mg bid. Interestingly, in a Thai study, lansoprazole 60 mg bid within a 10-d triple therapy showed a similar effectiveness of 10-d sequential therapy with lansoprazole at standard dose (80% *vs* 85%)[17]. At this regard, very high PPI doses have been frequently proposed in the context of dual therapy: A meta-analysis[18] demonstrated that such regimen, when used in rescue line, was equivalent to those recommended by guidelines (pooled eradication rate of 81.3% *vs* 81.5%, risk ratio = 1). In Taiwan, an optimized dual therapy with esomeprazole 40 mg tid plus amoxicillin 750 mg qid showed an effectiveness of the 91.7%, higher, even if not significantly (P = 0.21), than that of concomitant regimen, which displayed an outcome of the 86.7%[19]. Additionally, the same high-dose PPI-amoxicillin dual therapy achieved a very satisfactory eradication rate (96.1%) in China[20]. These recent evidences suggest that increasing PPI doses may have the power to reach an effectiveness similar to that obtained by adding further antibiotics[21]. Nevertheless, the real "test bed" of very high PPI dose is represented by the comparison with standard dose within the same eradication regimen. A meta-analysis[22] of seven studies, enrolling articles in which 7-d triple therapy was associated to either standard or very high dose, showed that the last one had a significantly higher eradication rate (82% versus 74%, with a risk ratio = 1.09, P = 0.03). Other similar head-to-head trials are summarized in Table 1[16,23-28]. As shown, in most cases the dose escalation allowed to gain a small increment in eradication rate without changes in adverse events[18]. Only one study, performed with dexlansoprazole, a new molecule, did not achieve a satisfactory eradication rate (53.8%) for dual therapy, but in this case only 13 patients were enrolled[29]. Finally, the utility of dose doubling has been underlined in a study by Ormeci *et al*[30] demonstrating that, in extensive metabolizers, triple therapy with standard dose of rabeprazole or pantoprazole failed in 12/75 and 13/81 patients respectively. Conversely, the re-administration of the same regimen with very high PPI dose in patients who failed therapy surprisingly allowed eradication in 10/12 for rabeprazole and 10/13 for pantoprazole. It is, therefore presumable, that in these cases treatment failure was not due to antibiotic resistance, but to an inadequate acid suppression.

PRE-TREATMENT WITH PPI

There is conflicting evidence whether a pretreatment with PPI may affect the efficacy of *H. pylori* eradicating regimens. Based on the assumption that PPIs slowly achieve the steady state and the optimal blood concentrations, it has been hypothesized that a pretreatment with these drugs before starting antibiotics may improve therapy effectiveness. Janssen *et al*. have reported that three-day pretreatment with a PPI (lansoprazole 30 mg twice daily) before quadruple eradication therapy paradoxically decreased eradication rate (66% *vs* 84%)[31]. The authors theorized that PPIs might induce coccoid-persistent forms of *H. pylori*, less vulnerable to the antibiotics. Other studies have reported that pretreatment did not affect treatment outcome[32-34]. Inoue *et al*. have reported that there was no statistically significant difference in the eradication rate with the regimen of lansoprazole, amoxicillin and clarithromycin between patients with and without pretreatment (lansoprazole 30 mg, once daily)[32]. A similar result was observed in a retrospective study by Tokoro *et al*[33]. The same Author investigated as well the influence of a pretreatment with histamine 2 receptor antagonist on *H. pylori* eradication; results indicated that bacterium eradication was not changed by this approach[34].

HAVE ALL PPI THE SAME EFFECTIVENESS?

The effectiveness of a PPI is related to the dose, formulation, relative power, frequency of administration and genetic differences in the activity of cytochrome P450 (CYP) enzymes of the subjects assuming the drug. A meta-analysis by McNicholl *et al*[35] compared first-generation (omeprazole, lansoprazole and pantoprazole) with second-generation PPIs (rabeprazole and esomeprazole). Thirty-five studies and 5998 patients were analyzed; the main finding of the meta-analysis was that the second-generation PPIs appeared to have a small superiority in eradication rate, because of their higher acid inhibition power. The eradication rates for esomeprazole was higher than for first-generation PPIs: 82.3% *vs* 77.6%; odd ratio (OR) = 1.32 [95% confidence interval (CI): 1.01-1.73]; also rabeprazole showed better results than first-generation

Table 1 Main studies exploring very high dose *vs* standard proton pump inhibitors dose in *Helicobacter pylori* eradication regimens

Study	PPI comparison	Antibiotic regimen	Very high dose eradication rate (%)	Standard dose eradication rate (%)
De Francesco *et al*[16], 2016	Esomeprazole 20 mg *vs* 40 mg bid	14-d triple therapy (CLA+AMO)	59/72 (81.9)	54/73 (73.9)
Gisbert *et al*[23], 2005	Esomeprazole 20 mg *vs* 40 mg bid	7-d triple therapy (CLA+AMO)	117/150 (78)	111/150 (74)
Anagnostopoulos *et al*[24], 2004	Omeprazole 20 mg *vs* esomeprazole 40mg bid	7-d triple therapy (CLA+AMO)	50/52 (96.1)	37/52 (71.1)
Sheu *et al*[25], 2005	Omeprazole 20 mg *vs* esomeprazole 40 mg bid	7-d triple therapy (CLA+AMO)	86/100 (86)	79/100 (79)
Manes *et al*[26], 2005	Omeprazole 20 mg *vs* omeprazole 40 mg bid	7-d triple therapy (CLA+TNZ)	132/161 (82)	135/162 (83.3)
Choi *et al*[27], 2007	Omeprazole 20 mg *vs* esomeprazole 40 mg bid	7-d triple therapy (CLA+AMO)	104/148 (70.3)	290/428 (67.7)
Hu *et al*[28], 2017	Rabeprazole 10 mg *vs* 20 mg qid	14-d dual therapy (AMO)	71/87 (81.6)	68/87 (78.1)

All percentages are expressed as intention to treat. AMO: Amoxicillin; CLA: Clarithromycin; TNZ: Tinidazole; PPI: Proton pump inhibitors.

PPIs: 80.5% *vs* 76.2%; OR = 1.21 (95%CI: 1.02-1.42). The new generation PPIs, esomeprazole and rabeprazole, had similar eradication rates: 78.7% *vs* 76.7%; OR = 0.90 (95%CI: 0.70-1.17).

Liver metabolism of PPIs is a relevant parameter since PPIs are prodrugs and, therefore, they are rapidly metabolized. The main enzymes involved in the metabolism of PPIs are CYP2C19 and CYP3A4. There are genetic differences in the activity of CYP2C19. Patients producing the largest amount of this enzyme are called "homozygous extensive metabolizer" (HomEM); "Heterozygous extensive metabolizer" (HetEM) are carriers of one wild-type and one mutation-type allele. Finally "poor metabolizer" (PM) patients have two "loss of function" variant alleles[36]. The pharmacokinetics properties of PPIs have significant difference between PM and HomEM. Indeed, in PM patients, a tendency towards a better eradication rate was found when treatment contained first-generation PPIs, whereas EM patients obtained higher eradication rates with therapy regimens based on new-generation PPIs[37]. Possible explanations may be that the CYP2C19 has no significant effect on the rabeprazole-based or esomeprazole-based triple therapies. Indeed, rabeprazole is metabolized through a non-enzymatic pathway, with scanty involvement of CYP2C19[38] and esomeprazole has only a minimal first pass metabolism characterized by poor hydroxylation via CYP2C19[39].

A meta-analysis by Padol *et al*[40] showed a significant difference between HetEM and HomEMs (OR = 1.90, 95%CI: 1.38-2.60, $P < 0.0001$) in favor of HetEM. A higher *H. pylori* eradication rates, in dual and triple omeprazole therapies, was recorded in PM over both HomEM (OR = 4.03, 95%CI: 1.97-8.28, $P = 0.0001$), and HetEM (OR = 2.24, 95%CI: 1.09-4.61, $P = 0.03$). Dual and triple rabeprazole and triple lansoprazole therapies were not significantly different between PM and HomEM (OR = 1.04, 95%CI: 0.44-2.46, $P = 0.25$)[40]. An additional meta-analysis of sixteen randomized clinical trials by Tang *et al*[41]. investigated the differences in eradication rate between HomEMs, HetEMs and PMs and confirmed the trend of a better eradication rate for PMs over HomEMs and HetEMs. The sub-analysis of individual PPIs showed a significant low eradication rate in both HomEMs versus HetEMs and HomEMs *vs* PMs with either omeprazole (OR 0.329; 95%CI: 0.195-0.553 and OR 0.232; 95%CI: 0.105-0.515, respectively) or lansoprazole (OR 0.692; 95%CI: 0.485-0.988 and OR 0.441; 95%CI: 0.252-0.771, respectively), while no significant difference was seen between HetEMs and PMs[41].

Additionally, some novel formulations of PPIs, such as modified release-dexlansoprazole, and instant release-omeprazole or tenatoprazole, which may increase the control of intragastric pH especially at night, are under investigation. However, they have not been tested yet for *H. pylori* despite they represent an excellent starting point for future investigations[42].

NOVEL DRUGS: A FOCUS ON VONOPRAZAN

Vonoprazan (VPZ) is a molecule that has been recently introduced in Japan for the

treatment of acid-related diseases, and it is currently available only in Japanese market[43]. VPZ is a competitive inhibitor of H+/K+-ATPase present on luminal membrane of gastric parietal cells. The binding power is much more powerful than conventional PPIs, *i.e.*, its biological activity is 300 times greater than lansoprazole[44,45]. VPZ has two further pharmacological benefits over other PPIs: It does not require pharmacological activation by gastric acid to inhibit acid secretion, and has a longer half-life, due to its slow dissociation kinetics from proton pump[46]. Another peculiarity consists in the rapid onset of the effect: While PPIs usually require more than 75-100 h to achieve the maximal gastric acid inhibitory effect[47,48], VPZ induces a fast, powerful and long-lasting gastric acid inhibition[49]. Therefore, in conclusion, VPZ seems to be better than conventional PPIs for five reasons: (1) It is more potent in acid secretion blockade; (2) It has a fast onset of action; (3) It is less prone to metabolization variables due to cytochrome polymorphisms; (4) Greater safety; and (5) Better tolerability[50].

VPZ is given at a dose of 20 mg bid in eradication regimens. A meta-analysis of ten studies[51], enrolling 10644 patients overall, demonstrated that triple therapy with VPZ in first line eradicated *H. pylori* in the 87.9%, while the eradication rate with traditional PPIs was 72.8% (risk ratio = 1.19, $P < 0.001$). Most of such studies were retrospective[52], however even randomized controlled trials, which are summarized in Table 2[53-55], confirmed that VPZ was better than conventional PPIs for *H. pylori* treatment in any case. However, the most interesting result comes from studies performed on clarithromycin resistant strains. A meta-analysis of 5 studies[56] showed that the eradication rate in clarithromycin susceptible patients did not differ from that of other PPIs (95.4% *vs* 92.8%), while VPZ was clearly superior in clarithromycin resistant strains (82% *vs* 40%, $P < 0.001$). Authors explained this finding with the following reasons: (1) VPZ prevents nocturnal acid breakthrough, which is linked to therapy failure; and (2) VPZ is not influenced by CYP2C19 polymorphisms. Finally, it seems that the effectiveness of this drug could be explained by its best suppression of acid secretion. Nevertheless, these enthusiastic results require to be confirmed by forthcoming studies outside Japan[50].

CONCLUSION

Evidence in literature clearly shows that a strong inhibition of gastric acid secretion is crucial for *H. pylori* eradication. Therefore, several tricks have to be used to optimize eradication rate of different regimens. The administration of the highest dose as possible seems to be able to improve therapeutic outcome even if specific trials are necessary. Pre-treatment does not seem to be effective, therefore it is discouraged. However, the choice of PPI molecule could have a certain weight, since second-generation substances are likely more effective because their metabolism is less dependent on CYP2C19 genetic variables. VPZ has shown the highest efficacy, but current data come from Eastern Asia, therefore its strong power needs to be confirmed outside this geographic area in Western countries. In the era of antibiotic resistance, the strategies to improve eradication rate are decreasing[57]. Until novel antibiotics will be discovered, a wise choice of antibiotic combination, may be guided by a tailored approach based on the knowledge of antibiotic susceptibility (preferably with non-invasive modality)[58-61]. Nevertheless, approaches which bypass the problem of antibiotic resistance, such as PPI optimization, vaccine, probiotics or phytochemistry, could be an useful ace up your sleeve[62-64]. In particular, the creation of novel and more effective and powerful PPIs could be an additional weapon against *H. pylori*. Finally, the marketing of VPZ in Western countries is expected to confirm its stunning results in the far East countries.

Table 2 Randomized controlled trials comparing vonoprazan *vs* proton pump inhibitors for *Helicobacter pylori* eradication regimens

Study	Protocol in vonoprazan group	Protocol in PPI group	Eradication rate in vonoprazan group	Eradication rate in PPI group
Murakami *et al*[53], 2016	Vonoprazan 20 mg bid	LAN 30 mg AMO 750 mg bid	299/329 (90.9%)	241/321 (75.1%)
	AMO 750 mg bid	CLA 400 mg bid		
	CLA 400 mg bid			
Maruyama *et al*[54], 2017	Vonoprazan 20 mg bid	LAN 30 mg or RAB 20 mg bid	69/72 (95.8%)	48/69 (69.6%)
	AMO 750 mg bid	AMO 750 mg bid		
	CLA 400 mg bid	CLA 400 mg bid		
Sue *et al*[55], 2018	Vonoprazan 20 mg bid	LAN 30 mg or RAB 10 mg or ESO 20 mg bid	48/55 (87.3%)	39/51 (76.5%)
	AMO 750 mg bid	AMO 750 mg bid		
	CLA 400 mg bid	CLA 400 mg bid		

All percentages are expressed as intention to treat. AMO: Amoxicillin; CLA: Clarithromycin; ESO: Esomeprazole; LAN: Lansoprazole; RAB: Rabeprazole; PPI: Proton pump inhibitors.

REFERENCES

1 **Ierardi E**, Goni E, Losurdo G, Di Mario F. Helicobacter pylori and nonmalignant diseases. *Helicobacter* 2014; **19** Suppl 1: 27-31 [PMID: 25167942 DOI: 10.1111/hel.12157]
2 **Waldum HL**, Kleveland PM, Sørdal ØF. Helicobacter pylori and gastric acid: An intimate and reciprocal relationship. *Therap Adv Gastroenterol* 2016; **9**: 836-844 [PMID: 27803738 DOI: 10.1177/1756283X16663395]
3 **Marshall BJ**. Virulence and pathogenicity of Helicobacter pylori. *J Gastroenterol Hepatol* 1991; **6**: 121-124 [PMID: 1912416 DOI: 10.1111/j.1440-1746.1991.tb01450.x]
4 **Keren I**, Kaldalu N, Spoering A, Wang Y, Lewis K. Persister cells and tolerance to antimicrobials. *FEMS Microbiol Lett* 2004; **230**: 13-18 [PMID: 14734160 DOI: 10.1016/S0378-1097(03)00856-5]
5 **Graham DY**, Shiotani A. New concepts of resistance in the treatment of Helicobacter pylori infections. *Nat Clin Pract Gastroenterol Hepatol* 2008; **5**: 321-331 [PMID: 18446147 DOI: 10.1038/ncpgasthep1138]
6 **Mizoguchi H**, Fujioka T, Nasu M. Evidence for viability of coccoid forms of Helicobacter pylori. *J Gastroenterol* 1999; **34** Suppl 11: 32-36 [PMID: 10616763 DOI: 10.1097/00004836-199912000-00014]
7 **Reshetnyak VI**, Reshetnyak TM. Significance of dormant forms of Helicobacter pylori in ulcerogenesis. *World J Gastroenterol* 2017; **23**: 4867-4878 [PMID: 28785141 DOI: 10.3748/wjg.v23.i27.4867]
8 **Graham DY**, Fischbach L. Helicobacter pylori treatment in the era of increasing antibiotic resistance. *Gut* 2010; **59**: 1143-1153 [PMID: 20525969 DOI: 10.1136/gut.2009.192757]
9 **Scott D**, Weeks D, Melchers K, Sachs G. The life and death of Helicobacter pylori. *Gut* 1998; **43** Suppl 1: S56-S60 [PMID: 9764042 DOI: 10.1136/gut.43.2008.S56]
10 **De Francesco V**, Zullo A, Perna F, Giorgio F, Hassan C, Vannella L, Cristofari F, Panella C, Vaira D, Ierardi E. Helicobacter pylori antibiotic resistance and [13C]urea breath test values. *J Med Microbiol* 2010; **59**: 588-591 [PMID: 20093375 DOI: 10.1099/jmm.0.018077-0]
11 **Maconi G**, Parente F, Russo A, Vago L, Imbesi V, Bianchi Porro G. Do some patients with Helicobacter pylori infection benefit from an extension to 2 weeks of a proton pump inhibitor-based triple eradication therapy? *Am J Gastroenterol* 2001; **96**: 359-366 [PMID: 11232676 DOI: 10.1111/j.1572-0241.2001.03519.x]
12 **Figura N**, Crabtree JE, Dattilo M. In-vitro activity of lansoprazole against Helicobacter pylori. *J Antimicrob Chemother* 1997; **39**: 585-590 [PMID: 9184356 DOI: 10.1093/jac/39.5.585]
13 **Malfertheiner P**, Megraud F, O'Morain CA, Gisbert JP, Kuipers EJ, Axon AT, Bazzoli F, Gasbarrini A, Atherton J, Graham DY, Hunt R, Moayyedi P, Rokkas T, Rugge M, Selgrad M, Suerbaum S, Sugano K, El-Omar EM; European Helicobacter and Microbiota Study Group and Consensus panel. Management of Helicobacter pylori infection-the Maastricht V/Florence Consensus Report. *Gut* 2017; **66**: 6-30 [PMID: 27707777 DOI: 10.1136/gutjnl-2016-312288]
14 **Zagari RM**, Romano M, Ojetti V, Stockbrugger R, Gullini S, Annibale B, Farinati F, Ierardi E, Maconi G, Rugge M, Calabrese C, Di Mario F, Luzza F, Pretolani S, Savio A, Gasbarrini G, Caselli M. Guidelines for the management of Helicobacter pylori infection in Italy: The III Working Group Consensus Report 2015. *Dig Liver Dis* 2015; **47**: 903-912 [PMID: 26253555 DOI: 10.1016/j.dld.2015.06.010]
15 **Molina-Infante J**, Lucendo AJ, Angueira T, Rodriguez-Tellez M, Perez-Aisa A, Balboa A, Barrio J, Martin-Noguerol E, Gomez-Rodriguez BJ, Botargues-Bote JM, Gomez-Camarero J, Huerta A, Modolell I, Arino I, Herranz-Bachiller MT, Bermejo F, McNicholl AG, O'Morain C, Gisbert JP, European Registry on H. pylori management (HpEuReg). Optimised empiric triple and concomitant therapy for Helicobacter pylori eradication in clinical practice: The OPTRICON study. *Aliment Pharmacol Ther* 2015; **41**: 581-589 [PMID: 25776067 DOI: 10.1111/apt.13069]
16 **De Francesco V**, Ridola L, Hassan C, Bellesia A, Alvaro D, Vaira D, Zullo A. Two-week Triple Therapy with either Standard or High-dose Esomeprazole for First-line H. pylori Eradication. *J Gastrointestin Liver Dis* 2016; **25**: 147-150 [PMID: 27308644 DOI: 10.15403/jgld.2014.1121.252.2w3]
17 **Auesomwang C**, Maneerattanaporn M, Chey WD, Kiratisin P, Leelakusolwong S, Tanwandee T. Ten-day high-dose proton pump inhibitor triple therapy versus sequential therapy for Helicobacter pylori eradication. *J Gastroenterol Hepatol* 2018; **33**: 1822-1828 [PMID: 29804294 DOI: 10.1111/jgh.14292]
18 **Gao CP**, Zhou Z, Wang JZ, Han SX, Li LP, Lu H. Efficacy and safety of high-dose dual therapy for

Helicobacter pylori rescue therapy: A systematic review and meta-analysis. *J Dig Dis* 2016; **17**: 811-819 [PMID: 27977071 DOI: 10.1111/1751-2980.12432]
19 **Tai WC**, Liang CM, Kuo CM, Huang PY, Wu CK, Yang SC, Kuo YH, Lin MT, Lee CH, Hsu CN, Wu KL, Hu TH, Chuah SK. A 14 day esomeprazole- and amoxicillin-containing high-dose dual therapy regimen achieves a high eradication rate as first-line anti-Helicobacter pylori treatment in Taiwan: A prospective randomized trial. *J Antimicrob Chemother* 2019; **74**: 1718-1724 [PMID: 30768161 DOI: 10.1093/jac/dkz046]
20 **Yu L**, Luo L, Long X, Liang X, Ji Y, Graham DY, Lu H. High-dose PPI-amoxicillin dual therapy with or without bismuth for first-line Helicobacter pylori therapy: A randomized trial. *Helicobacter* 2019; **24**: e12596 [PMID: 31111580 DOI: 10.1111/hel.12596]
21 **Gisbert JP**, McNicholl AG. Optimization strategies aimed to increase the efficacy of H. pylori eradication therapies. *Helicobacter* 2017; 22 [PMID: 28464347 DOI: 10.1111/hel.12392]
22 **Villoria A**, Garcia P, Calvet X, Gisbert JP, Vergara M. Meta-analysis: High-dose proton pump inhibitors vs. standard dose in triple therapy for Helicobacter pylori eradication. *Aliment Pharmacol Ther* 2008; **28**: 868-877 [PMID: 18644011 DOI: 10.1111/j.1365-2036.2008.03807.x]
23 **Gisbert JP**, Domínguez-Muñoz A, Domínguez-Martín A, Gisbert JL, Marcos S. Esomeprazole-based therapy in Helicobacter pylori eradication: Any effect by increasing the dose of esomeprazole or prolonging the treatment? *Am J Gastroenterol* 2005; **100**: 1935-1940 [PMID: 16128936 DOI: 10.1111/j.1572-0241.2005.00178.x]
24 **Anagnostopoulos GK**, Tsiakos S, Margantinis G, Kostopoulos P, Arvanitidis D. Esomeprazole versus omeprazole for the eradication of Helicobacter pylori infection: Results of a randomized controlled study. *J Clin Gastroenterol* 2004; **38**: 503-506 [PMID: 15220685 DOI: 10.1097/01.mcg.0000129061.54277.c6]
25 **Sheu BS**, Kao AW, Cheng HC, Hunag SF, Chen TW, Lu CC, Wu JJ. Esomeprazole 40 mg twice daily in triple therapy and the efficacy of Helicobacter pylori eradication related to CYP2C19 metabolism. *Aliment Pharmacol Ther* 2005; **21**: 283-288 [PMID: 15691303 DOI: 10.1111/j.1365-2036.2005.02281.x]
26 **Manes G**, Pieramico O, Perri F, Vaira D, Giardullo N, Romano M, Nardone G, Balzano A. Twice-daily standard dose of omeprazole achieves the necessary level of acid inhibition for Helicobacter pylori eradication. A randomized controlled trial using standard and double doses of omeprazole in triple therapy. *Dig Dis Sci* 2005; **50**: 443-448 [PMID: 15810623 DOI: 10.1007/s10620-005-2455-6]
27 **Choi HS**, Park DI, Hwang SJ, Park JS, Kim HJ, Cho YK, Sohn CI, Jeon WK, Kim BI. Double-dose, new-generation proton pump inhibitors do not improve Helicobacter pylori eradication rate. *Helicobacter* 2007; **12**: 638-642 [PMID: 18001407 DOI: 10.1111/j.1523-5378.2007.00556.x]
28 **Hu JL**, Yang J, Zhou YB, Li P, Han R, Fang DC. Optimized high-dose amoxicillin-proton-pump inhibitor dual therapies fail to achieve high cure rates in China. *Saudi J Gastroenterol* 2017; **23**: 275-280 [PMID: 28937021 DOI: 10.4103/sjg.SJG_91_17]
29 **Attumi TA**, Graham DY. High-dose extended-release lansoprazole (dexlansoprazole) and amoxicillin dual therapy for Helicobacter pylori infections. *Helicobacter* 2014; **19**: 319-322 [PMID: 24698653 DOI: 10.1111/hel.12126]
30 **Ormeci A**, Emrence Z, Baran B, Soyer OM, Gokturk S, Evirgen S, Akyuz F, Karaca C, Besisik F, Kaymakoglu S, Ustek D, Demir K. Can Helicobacter pylori be eradicated with high-dose proton pump inhibitor in extensive metabolizers with the CYP2C19 genotypic polymorphism? *Eur Rev Med Pharmacol Sci* 2016; **20**: 1795-1797 [PMID: 27212172]
31 **Janssen MJ**, Laheij RJ, Jansen JB, de Boer WA. The influence of pretreatment on cure rates of Helicobacter pylori eradication. *Neth J Med* 2004; **62**: 192-196 [PMID: 15460498 DOI: 10.1089/1540999041281052]
32 **Inoue M**, Okada H, Hori S, Kawahara Y, Kawano S, Takenaka R, Toyokawa T, Onishi Y, Shiratori Y, Yamamoto K. Does pretreatment with lansoprazole influence Helicobacter pylori eradication rate and quality of life? *Digestion* 2010; **81**: 218-222 [PMID: 20090337 DOI: 10.1159/000260416]
33 **Tokoro C**, Inamori M, Koide T, Iida H, Sakamoto Y, Endo H, Hosono K, Takahashi H, Yoneda M, Yasuzaki H, Ogawa M, Abe Y, Kubota K, Saitou S, Kawana I, Matsuda R, Takahashi D, Nakajima A. Does pretreatment with proton pump inhibitors influence the eradication rate of Helicobacter pylori? *Hepatogastroenterology* 2010; **57**: 1645-1649 [PMID: 21443136 DOI: 10.1007/s11999-010-1543-x]
34 **Tokoro C**, Inamori M, Koide T, Sekino Y, Iida H, Sakamoto Y, Endo H, Hosono K, Takahashi H, Yoneda M, Yasuzaki H, Ogawa M, Abe Y, Kubota K, Saito S, Kawana I, Nakajima A, Maeda S, Matsuda R, Takahashi D. Influence of pretreatment with H2 receptor antagonists on the cure rates of Helicobacter pylori eradication. *Med Sci Monit* 2011; **17**: CR235-CR240 [PMID: 21525804 DOI: 10.12659/MSM.881762]
35 **McNicholl AG**, Linares PM, Nyssen OP, Calvet X, Gisbert JP. Meta-analysis: Esomeprazole or rabeprazole vs. first-generation pump inhibitors in the treatment of Helicobacter pylori infection. *Aliment Pharmacol Ther* 2012; **36**: 414-425 [PMID: 22803691 DOI: 10.1111/j.1365-2036.2012.05211.x]
36 **Tomalik-Scharte D**, Lazar A, Fuhr U, Kirchheiner J. The clinical role of genetic polymorphisms in drug-metabolizing enzymes. *Pharmacogenomics J* 2008; **8**: 4-15 [PMID: 17549068 DOI: 10.1038/sj.tpj.6500462]
37 **Kuo CH**, Lu CY, Shih HY, Liu CJ, Wu MC, Hu HM, Hsu WH, Yu FJ, Wu DC, Kuo FC. CYP2C19 polymorphism influences Helicobacter pylori eradication. *World J Gastroenterol* 2014; **20**: 16029-16036 [PMID: 25473155 DOI: 10.3748/wjg.v20.i43.16029]
38 **Adachi K**, Katsube T, Kawamura A, Takashima T, Yuki M, Amano K, Ishihara S, Fukuda R, Watanabe M, Kinoshita Y. CYP2C19 genotype status and intragastric pH during dosing with lansoprazole or rabeprazole. *Aliment Pharmacol Ther* 2000; **14**: 1259-1266 [PMID: 11012469 DOI: 10.1046/j.1365-2036.2000.00840.x]
39 **Andersson T**, Hassan-Alin M, Hasselgren G, Röhss K, Weidolf L. Pharmacokinetic studies with esomeprazole, the (S)-isomer of omeprazole. *Clin Pharmacokinet* 2001; **40**: 411-426 [PMID: 11475467 DOI: 10.2165/00003088-200140060-00003]
40 **Padol S**, Yuan Y, Thabane M, Padol IT, Hunt RH. The effect of CYP2C19 polymorphisms on H. pylori eradication rate in dual and triple first-line PPI therapies: A meta-analysis. *Am J Gastroenterol* 2006; **101**: 1467-1475 [PMID: 16863547 DOI: 10.1111/j.1572-0241.2006.00717.x]
41 **Tang HL**, Li Y, Hu YF, Xie HG, Zhai SD. Effects of CYP2C19 loss-of-function variants on the eradication of H. pylori infection in patients treated with proton pump inhibitor-based triple therapy regimens: A meta-analysis of randomized clinical trials. *PLoS One* 2013; **8**: e62162 [PMID: 23646118 DOI: 10.1371/journal.pone.0062162]
42 **Hunt RH**, Scarpignato C. Potent Acid Suppression with PPIs and P-CABs: What's New? *Curr Treat*

Options Gastroenterol 2018; **16**: 570-590 [PMID: 30361857 DOI: 10.1371/journal.pone.0062162]
43 **Mori H**, Suzuki H. Role of Acid Suppression in Acid-related Diseases: Proton Pump Inhibitor and Potassium-competitive Acid Blocker. *J Neurogastroenterol Motil* 2019; **25**: 6-14 [PMID: 30504527 DOI: 10.5056/jnm18139]
44 **Parsons ME**, Keeling DJ. Novel approaches to the pharmacological blockade of gastric acid secretion. *Expert Opin Investig Drugs* 2005; **14**: 411-421 [PMID: 15882117 DOI: 10.1517/13543784.14.4.411]
45 **Ashida K**, Sakurai Y, Hori T, Kudou K, Nishimura A, Hiramatsu N, Umegaki E, Iwakiri K. Randomised clinical trial: Vonoprazan, a novel potassium-competitive acid blocker, vs. lansoprazole for the healing of erosive oesophagitis. *Aliment Pharmacol Ther* 2016; **43**: 240-251 [PMID: 26559637 DOI: 10.1111/apt.13461]
46 **Scott DR**, Munson KB, Marcus EA, Lambrecht NW, Sachs G. The binding selectivity of vonoprazan (TAK-438) to the gastric H+, K+ -ATPase. *Aliment Pharmacol Ther* 2015; **42**: 1315-1326 [PMID: 26423447 DOI: 10.1111/apt.13414]
47 **Saitoh T**, Fukushima Y, Otsuka H, Hirakawa J, Mori H, Asano T, Ishikawa T, Katsube T, Ogawa K, Ohkawa S. Effects of rabeprazole, lansoprazole and omeprazole on intragastric pH in CYP2C19 extensive metabolizers. *Aliment Pharmacol Ther* 2002; **16**: 1811-1817 [PMID: 12269976 DOI: 10.1046/j.1365-2036.2002.01348.x]
48 **Sugimoto M**, Furuta T, Shirai N, Ikuma M, Hishida A, Ishizaki T. Initial 48-hour acid inhibition by intravenous infusion of omeprazole, famotidine, or both in relation to cytochrome P450 2C19 genotype status. *Clin Pharmacol Ther* 2006; **80**: 539-548 [PMID: 17112810 DOI: 10.1016/j.clpt.2006.08.010]
49 **Jenkins H**, Sakurai Y, Nishimura A, Okamoto H, Hibberd M, Jenkins R, Yoneyama T, Ashida K, Ogama Y, Warrington S. Randomised clinical trial: Safety, tolerability, pharmacokinetics and pharmacodynamics of repeated doses of TAK-438 (vonoprazan), a novel potassium-competitive acid blocker, in healthy male subjects. *Aliment Pharmacol Ther* 2015; **41**: 636-648 [PMID: 25707624 DOI: 10.1111/apt.13121]
50 **Abadi ATB**, Ierardi E. Vonoprazan and Helicobacter pylori Treatment: A Lesson From Japan or a Limited Geographic Phenomenon? *Front Pharmacol* 2019; **10**: 316 [PMID: 31024299 DOI: 10.3389/fphar.2019.00316]
51 **Jung YS**, Kim EH, Park CH. Systematic review with meta-analysis: The efficacy of vonoprazan-based triple therapy on Helicobacter pylori eradication. *Aliment Pharmacol Ther* 2017; **46**: 106-114 [PMID: 28497487 DOI: 10.1111/apt.14130]
52 **Sugimoto M**, Yamaoka Y. Role of Vonoprazan in Helicobacter pylori Eradication Therapy in Japan. *Front Pharmacol* 2019; **9**: 1560 [PMID: 30697158 DOI: 10.3389/fphar.2018.01560]
53 **Murakami K**, Sakurai Y, Shiino M, Funao N, Nishimura A, Asaka M. Vonoprazan, a novel potassium-competitive acid blocker, as a component of first-line and second-line triple therapy for Helicobacter pylori eradication: A phase III, randomised, double-blind study. *Gut* 2016; **65**: 1439-1446 [PMID: 26935876 DOI: 10.1136/gutjnl-2015-311304]
54 **Maruyama M**, Tanaka N, Kubota D, Miyajima M, Kimura T, Tokutake K, Imai R, Fujisawa T, Mori H, Matsuda Y, Wada S, Horiuchi A, Kiyosawa K. Vonoprazan-Based Regimen Is More Useful than PPI-Based One as a First-Line Helicobacter pylori Eradication: A Randomized Controlled Trial. *Can J Gastroenterol Hepatol* 2017; **2017**: 4385161 [PMID: 28349044 DOI: 10.1155/2017/4385161]
55 **Sue S**, Ogushi M, Arima I, Kuwashima H, Nakao S, Naito M, Komatsu K, Kaneko H, Tamura T, Sasaki T, Kondo M, Shibata W, Maeda S. Vonoprazan- vs proton-pump inhibitor-based first-line 7-day triple therapy for clarithromycin-susceptible Helicobacter pylori: A multicenter, prospective, randomized trial. *Helicobacter* 2018; **23**: e12456 [PMID: 29271026 DOI: 10.1111/hel.12456]
56 **Li M**, Oshima T, Horikawa T, Tozawa K, Tomita T, Fukui H, Watari J, Miwa H. Systematic review with meta-analysis: Vonoprazan, a potent acid blocker, is superior to proton-pump inhibitors for eradication of clarithromycin-resistant strains of Helicobacter pylori. *Helicobacter* 2018; **23**: e12495 [PMID: 29873436 DOI: 10.1111/hel.12495]
57 **Ierardi E**, Giorgio F, Losurdo G, Di Leo A, Principi M. How antibiotic resistances could change Helicobacter pylori treatment: A matter of geography? *World J Gastroenterol* 2013; **19**: 8168-8180 [PMID: 24363506 DOI: 10.3748/wjg.v19.i45.8168]
58 **Iannone A**, Giorgio F, Russo F, Riezzo G, Girardi B, Pricci M, Palmer SC, Barone M, Principi M, Strippoli GF, Di Leo A, Ierardi E. New fecal test for non-invasive Helicobacter pylori detection: A diagnostic accuracy study. *World J Gastroenterol* 2018; **24**: 3021-3029 [PMID: 30038469 DOI: 10.3748/wjg.v24.i27.3021]
59 **Ierardi E**, Giorgio F, Iannone A, Losurdo G, Principi M, Barone M, Pisani A, Di Leo A. Noninvasive molecular analysis of Helicobacter pylori: Is it time for tailored first-line therapy? *World J Gastroenterol* 2017; **23**: 2453-2458 [PMID: 28465629 DOI: 10.3748/wjg.v23.i14.2453]
60 **Giorgio F**, Ierardi E, Sorrentino C, Principi M, Barone M, Losurdo G, Iannone A, Giangaspero A, Monno R, Di Leo A. Helicobacter pylori DNA isolation in the stool: An essential pre-requisite for bacterial noninvasive molecular analysis. *Scand J Gastroenterol* 2016; **51**: 1429-1432 [PMID: 27687850 DOI: 10.1080/00365521.2016.1216592]
61 **Cammarota G**, Ianiro G, Bibbò S, Di Rienzo TA, Masucci L, Sanguinetti M, Gasbarrini A. Culture-guided treatment approach for Helicobacter pylori infection: Review of the literature. *World J Gastroenterol* 2014; **20**: 5205-5211 [PMID: 24833850 DOI: 10.3748/wjg.v20.i18.5205]
62 **Talebi Bezmin Abadi A**. Vaccine against Helicobacter pylori: Inevitable approach. *World J Gastroenterol* 2016; **22**: 3150-3157 [PMID: 27003991 DOI: 10.3748/wjg.v22.i11.3150]
63 **Losurdo G**, Cubisino R, Barone M, Principi M, Leandro G, Ierardi E, Di Leo A. Probiotic monotherapy and Helicobacter pylori eradication: A systematic review with pooled-data analysis. *World J Gastroenterol* 2018; **24**: 139-149 [PMID: 29358890 DOI: 10.3748/wjg.v24.i1.139]
64 **Vale FF**, Oleastro M. Overview of the phytomedicine approaches against Helicobacter pylori. *World J Gastroenterol* 2014; **20**: 5594-5609 [PMID: 24914319 DOI: 10.3748/wjg.v20.i19.5594]

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ORIGINAL ARTICLE

Basic Study

Regulatory effect of a Chinese herbal medicine formula on non-alcoholic fatty liver disease

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Abstract

BACKGROUND

Non-alcoholic fatty liver disease (NAFLD) has become a major cause of chronic liver disease. The Chinese herbal medicine (CHM) Dachaihu decoction (DCHD) has been proved to treat NAFLD with good efficacy in previous studies. Based on the TCM principle of formula formation, we divided DCHD into soothing liver part, invigorating spleen part, and dredging intestine part. Marshall officially proposed the concept of "intestinal-hepatic axis", which systematically explains the interactions between the intestine and liver. We hypothesized that the effect of CHM on NAFLD is achieved by regulating the liver and intestine. Thus, we aimed to investigate the possible effect of a CHM formula on NAFLD in a rat model.

AIM

To investigate the effects of a CHM formula (a decoction of Chinese thorowax root, scutellaria root, and white peony root) on NAFLD and its regulatory effect on the "intestinal-liver" axis.

METHODS

Sixty rats were randomly divided into control, model, pioglitazone hydrochloride (PH), and CHM (a decoction of Chinese thorowax root, scutellaria root, and white peony root) groups. An NAFLD rat model was established using a high-fat high-fructose diet for 16 wk. From the 13th week, rats were administered with PH or a decoction of Chinese thorowax, scutellaria, and white peony root (CHM group) for 4 wk. Rats in the control group and model group were administered with an equal volume of distilled water. At the end of the study, blood was collected *via* the abdominal aorta. Liver tissues were harvested and any morphological changes were observed by hematoxylin-eosin (HE) staining, Oil red O staining,

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and Masson staining. In addition, blood lipids, liver function markers, and triglyceride (TG) in liver tissues were analyzed. The levels of transforming growth factor-β1 (TGF-β1), tumor necrosis factor-α (TNF-α), Toll-like receptor-4 (TLR4), and nuclear factor-kappa B (NF-κB) in liver tissues and secreted immunoglobulin A (sIgA) in intestinal tissues were analyzed by ELISA, and protein and mRNA expression of occludin and zonula occludens-1 (ZO-1) in the intestine were measured using Western blot and reverse transcription-quantitative polymerase chain reaction, respectively. The endotoxin level in plasma was detected by endpoint chromogenic assay.

RESULTS

Compared to the normal control group, the liver coefficient, serum TG, total cholesterol (TC), low density lipoprotein (LDL), aspartate aminotransferase (AST), and alanine aminotransferase (ALT), blood glucose, plasma endotoxin, and the levels of TG, TNF-α, TGF-β, NF-kB, and TLR4 in liver tissues increased significantly in the model group, while serum high density lipoprotein (HDL), intestinal sIgA, and protein and mRNA expression of occludin and ZO-1 decreased significantly in the model group ($P < 0.01$). PH and CHM attenuated the elevated liver coefficient, serum TG, TC, LDL, AST, and ALT, blood glucose, plasma endotoxin, and the levels of TG, TNF-α, TGF-β, NF-kB, and TLR4 in liver tissues and increased serum HDL levels compared to the model group ($P < 0.01$). Intestinal sIgA and the protein and mRNA expression of intestinal occludin and ZO-1 were significantly increased in the PH group compared to the model and CHM groups ($P < 0.01$).

CONCLUSION

The decoction of Chinese thorowax root, scutellaria root, and white peony root is beneficial in regulating lipid metabolism and liver function, which indicates that it has a good effect on the liver. To a certain extent, this CHM formula can affect both the liver and intestine, while its effect on the liver is superior to that on the intestine.

Key words: Non-alcoholic fatty liver disease; Chinese herbal medicine; Liver function; Intestinal-hepatic axis

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Core tip: Based on the previous study about the "intestinal-hepatic axis", we hypothesized that the effect of Chinese herbal medicine (CHM) on non-alcoholic fatty liver disease is achieved by regulating the liver and intestine. In this study, we found that a CHM formula (a decoction of thorowax root, scutellaria root, and white peony root) had a good effect in regulating lipid metabolism and liver function, which suggested its ability to regulate the liver. To a certain extent, it also had a regulatory effect on the intestinal mucosal barrier. Our study demonstrated that the CHM formula can affect both the liver and intestine, while its effect on the liver is superior to that on the intestine.

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INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is one of the manifestations of fatty liver and occurs when fat is deposited (steatosis) in the liver due to causes other than excessive alcohol use[1]. The incidence of NAFLD has increased over the years with a prevalence of 25%-30% in the general population. This makes it one of the most major causes of chronic liver disease and affects both adults and children. NAFLD is more common in patients with severe diabetes and obesity and has gained more attention

worldwide[2,3]. Hepatic morphology and functionality in NAFLD patients are adversely affected. These adverse effects are categorized into four different stages, starting from simple steatosis (liver fat deposition and mild inflammation), development of non-alcoholic steatohepatitis (NASH) that includes steatosis plus inflammation and hepatocyte "ballooning", the appearance of fibrosis and, in some cases, leading to late stage hepatocellular carcinoma (HCC). Although the exact mechanisms leading to NAFLD are not fully deciphered, insulin resistance, hormones secreted from adipose tissues, nutritional factors, gut microbiota, and genetic/epigenetic factors have been suggested to play a major role[4]. Approximately 90% of patients with NAFLD show a close association with one or more of the following risk factors: Hypertension, dyslipidemia, elevated triglyceride (TG) levels, obesity, insulin resistance, metabolic syndrome, type 2 diabetes mellitus, and cardiovascular disease[1]. There are currently no effective treatment options for NAFLD, with the exemption of lifestyle changes[5].

The pathogenesis of NAFLD remains unclear; however, the "two-hit" hypothesis is the most widely accepted theory, which states that fat deposition in the liver represents the "first hit", and increased levels of oxidative stress, insulin resistance, and inflammatory cytokines induced by fat deposition represents the "second hit"[6]. In addition, the "multi-hit" theory has been proposed, which states that several extrahepatic factors, such as adipose tissue and the intestinal tract participate in accelerating the occurrence of liver inflammation and the development of NAFLD[7]. In 1998, Marshall officially proposed the concept of "intestinal-hepatic axis", which systematically explains the interactions between the intestine and liver and how they interact and regulate substances, cells, and cytokines and each other.

Chinese herbal medicine (CHM) has been traditionally used in China and other Asian countries for thousands of years. A specific and basic feature of Chinese medicine is the use of formulas containing several herbs (herbal cocktail) to ameliorate abnormal symptoms associated with a particular disease[8]. Dachaihu decoction (DCHD), a classical formula from the *Treatise on Febrile Disease*, is one of the well-known traditional Chinese medicines and consists of: Chinese thorowax root (*Bupleurum chinensis DC.*), scutellaria root (*Scutellaria baicalensis Georgi*), white peony root (*Paeonia tacti lora Pall*), prepared pinellia tuber (*Pinellia ternate*), fresh ginger (*Zingiber officinale Roscoe*), Chinese date (*Ziziphus jujube MILL.*), rhubarb root and rhizome (*Rheum palmatum L.*), and immature bitter orange (*Citrus aurantium L.*)[9]. Previous studies have demonstrated these TCM formulas had good efficacy in treating NAFLD[10,11]. Based on the TCM principle of formula formation, we divided DCHD into three parts; part 1 is "soothing liver" (Chinese thorowax root, scutellaria root, and white peony root), which has a good effect in regulating the liver; part 2 is "invigorating spleen" (prepared pinellia tuber, fresh ginger, and Chinese date), which has a good effect in regulating the spleen, and part 3 is "dredging intestine" (rhubarb root and rhizome and immature bitter orange), which has an effect in dredging the turbidity and stool.

The aim of the present study was to investigate the therapeutic effects of the soothing liver herbs of DCHD on NAFLD in a rat model. We hypothesized that the effect of CHM on NAFLD may be achieved by regulating the intestinal-hepatic axis. Whether the regulatory effect is mainly on the liver or the intestine needs experimental observation. To test our hypothesis, we measured the liver weight/body weight ratio, liver histopathology, serum liver enzymes, cholesterol lipoproteins, cytokines, and gene and protein expression in the intestine in a rate model of NAFLD.

MATERIALS AND METHODS

Experimental animals

A total of 60 male Sprague-Dawley (SD) rats weighing 180 ± 20 g were purchased from SPF (Beijing) Biotechnology [License No: SCXK (2016-0002; Beijing, China]. Rats were housed at a constant temperature of 23 °C ± 1 °C, a constant humidity of 45p.100 ± 5p.100, with 12 h light/dark cycles. Water and laboratory rodent chow were provided *ad libitum*. This study was approved by the Medical and Experimental Animal Ethics Committee of Beijing University of Chinese Medicine (BUCM-1-2017051030-2030).

Drug and decoction preparation

Pioglitazone hydrochloride (PH) tablets were used as the positive control and was purchased from Jingdong Pharmacy (Huadong Medicine, product lot number: 170102). Based on a previous study[12], pioglitazone was administered at a dosage of 10 mg/kg per day. Tablets were ground into a powder and dissolved in distilled water.

The prepared suspension was refrigerated and dispensed when required.

TCM herbs bupleurum, scutellaria roots, and white peony roots were purchased from Beijing Tong Ren Tang Pharmacy (Beijing, China). Their dosages were as follows: Chinese thorowax root (*Bupleurum chinense* DC = 15 g), scutellaria root (*Scutellaria baicalensis georgi* = 9 g), and white peony root (*Paeonia lactiflora* PALL = 9 g). Based on body surface area and human-rat dose conversion, the dosage of the crude drugs for rats was set at 8.64 g/kg[13].

Initially the ingredients were placed in an earthenware pot and distilled water was added until all the ingredients were covered. The ingredients were soaked for 30 min and then the solution was boiled and simmered. The decoction was filtered, and more water was added to the remaining ingredients and then boiled again as described previously. The two decoctions were mixed and the final concentration of the herbal medicinal extracts was 1 g/mL. The decoction was stored in a refrigerator until used.

Experimental design

Sixty SD rats were randomly and equally divided into four groups: Control, model, PH, and CHM groups. The control group was fed a standard diet (n = 15), while the other three groups (model group, PH group, and CHM group) were fed a high-fat high-fructose diet containing 52.5% basic food, 2% cholesterol, 10% lard, 5% egg yolk powder, 0.5% sodium cholate, and 30% sucrose. The calories percent of the fat in the diet was 11.6%, and the calories percent of carbohydrates in the diet was 65.4%. The rats were housed in a temperature-controlled environment with 50% humidity and fed for 12 wk. From the 13th week, rats in the PH group were administered intragastrically with PH at a dose of 10 mg/kg/d (n = 8) for 4 wk and fed the high-fat high-fructose diet. Rats in the CHM group were administered intragastrically with thorowax root, scutellaria root, and white peony root decoction for 4 wk and fed the high-fat high-fructose diet. The normal group and model group were provided with an equal volume of distilled water and fed the standard diet and high-fat high-fructose diet, respectively.

Sample collection

After 16 wk of feeding, all rats were fasted for 12 h and eight of them from each group were randomly selected for blood and liver tissue collection. Blood samples from the tail tip were obtained for blood sugar detection. At the end of the study, the animals were anesthetized and abdominal blood samples from all rats were collected for serum biochemical assays. Liver tissues from the same region for all rats were fixed in 4% paraformaldehyde, paraffin-embedded, and sectioned. The remaining liver sections were stored at -80°C until needed for other assays.

Calculation of liver coefficient

At the end of the experiment, body weight and liver weight of all rats for the four groups were measured and recorded. Liver coefficients were calculated as absolute liver weight (g)/body weight of rat on sacrifice day (g) × 100%.

Histopathological analysis by hematoxylin and eosin (HE) staining

Liver tissues were fixed in 4% paraformaldehyde and then embedded in paraffin and sliced into 5 μm thick sections. The sections were then stained with HE for morphological examination using a light microscope (BX-53; Olympus) at 200× magnification.

Oil red O (ORO) staining

Frozen hepatic tissues were cut at 6 μm and mounted on slides, air-dried, and then fixed in ice-cold 10% formaldehyde solution for 10 min. Slides were rinsed immediately in distilled water for several seconds and then placed in isopropanol solution for 20-30 s. Slides were then stained in ORO solution for 15-20 min, and then rinsed in distilled water prior to staining with hematoxylin for 40 s. After washing thoroughly in running tap water for 5 min, the slides were placed in distilled water and then mounted with glycerin jelly. The HE- and ORO-stained slides were visualized using a light microscope to visualize the architecture of the liver and hepatic lipid droplets. Image processing software pro plus 6.0 (Media Cybernetics, Maryland, United States) was used for data quantification. Liver lipid droplet vacuolar area ratio was calculated as lipid droplet area/total area of the picture × 100%.

Masson's trichrome staining

Liver tissues were fixed in 4% paraformaldehyde, embedded in paraffin, and then sectioned. Slides were then hydrated through a series of graded alcohols (100, 95, 90, 80, and 70%) for 5 min each. The slides were then stained with Masson's trichrome

dye.

Assays for serum biochemical markers

Abdominal aortic blood was collected and serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), total cholesterol (TC), TG, high density lipoprotein-cholesterol (HDL-C), and low-density lipoprotein cholesterol (LDL-C) levels were measured using an automatic biochemical detector from Beckman Coulter Inc. (California, United States).

Measurements of cytokines and protein expression in liver tissue and secreted immunoglobulin A (sIgA) in intestine tissue

A 100 mg liver tissue piece and intestine tissue piece from the same portion were placed into a 1.5 mL tube, 1 mL of 0.9% saline was added, and the piece was cut into smaller pieces. After grinding, the supernatant was absorbed after centrifugation for 15 min (4 °C, 1500 rpm). Enzyme-linked immunosorbent assay (ELISA) kits were used to test the levels of each protein according to the instructions provided. The levels of tumor necrosis factor (TNF-α) and transforming growth factor (TGF-β1) in liver tissues and sIgA in intestinal tissues were measured using ELISA kits (NeoBioscience, Shenzhen, China). The levels of nuclear factor-kappa B (NF-κB) and Toll-like receptor (TLR4) in liver tissues were measured using a commercial kit (USCN Life Science, Wuhan, China). The TG level in liver tissue was measured using an automatic biochemical detector from Beckman Coulter Inc. (California, United States).

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted using TRIZOL reagent according to the manufacturer's instructions. The concentration and purity of the RNA samples were measured. cDNA was synthesized for RT-PCR amplification according to the instructions in the First Strand cDNA Synthesis Kit (Invitrogen). The sequences of the primers used for the RT-PCR assay are shown in Table 1. The reverse transcription conditions were as follows: 37 °C for 15 min, followed by 5 s at 85 °C for RT inactivation. qPCR was performed using the following conditions: 30s at 95 °C for denaturation; 5 s at 95 °C for annealing, and 40 s at 60 °C for extension.

Western blot analysis

Frozen intestine tissues were homogenized in ice-cold PIPA lysis buffer and then centrifuged at 12000 rpm for 15 min. The supernatants were collected and used for Western blot analysis. Total protein concentration was determined using the BCA protein assay kit [Beijing Pulilai Gene Technology Co., Ltd. (Beijing, China)]. Equal amounts of protein samples were resolved by 12% SDS-PAGE and transferred to polyvinylidene difluoride (PVDV) membranes (Millipore, United States). The membranes were blocked at room temperature with 5% dried skimmed milk for 1 h and then incubated at 4 °C overnight with different primary antibodies: ZO-1 (Proteintech), occludin (Abcam), and β-actin (Proteintech). Afterwards, the membranes were washed and incubated for 1 h with secondary antibodies at room temperature. Protein bands were visualized using a LAS-4000MINI Biomolecular imager (BIO-RAD, United States). Protein amounts were quantified using Quantity One (BIO-RAD, United States).

Plasma endotoxin level measurement

Blood was collected from the abdominal aorta and centrifuged at 2500 rpm at 4 °C for 5 min. The supernatant was transferred to a non-pyrogenic centrifuge tube. The endotoxin level in plasma was detected with an endpoint chromogenic assay from Chinese Horseshoe Crab Reagent Manufactory Co., Ltd. (Xiamen, China).

Statistical analysis

All data are expressed as the mean ± SD. SPSS17.0 software package (IBM, Armonk, NY, United States) was used for statistical analyses. Homogeneity of variance test was performed. Then, the data were analyzed using a one-way ANOVA test or Welch test and a post hoc test. $P < 0.05$ was considered statistically significant.

RESULTS

Effect of CHM on liver coefficient

After feeding a high-fat high-fructose diet for 16 wk, the liver coefficient increased significantly in the model group compared to the control group ($P < 0.01$). Compared to the model group, the liver coefficient was significantly reduced in the PH and CHM groups ($P < 0.01$). The liver coefficient in the PH group was lower compared to

Table 1 Primers used for RT-qPCR

Gene	Forward	Reverse
ZO-1	5′- CTGATGGTGTTCTGCCAAATTC – 3′	5' - GTCGCAAACCCACACTATCT - 3'
Occludin	5′- CCATCTGACTATGCGGAAAGAG – 3′	5' - TACCAGAGGCGGTGACTTAT - 3'
β-actin	5′ - ACCGTGAAAAGATGACCCAGAT – 3′	5' - CCAGAGGCATACAGGGACAA - 3'

ZO-1: Zonula occludens-1.

that of the CHM group ($P < 0.01$) (Table 2).

Changes in liver histopathology

HE-stained sections are shown in Figure 1. Liver sections from the normal control group had intact and clear hepatic lobule structures, the boundary of the portal area was clear, normal hepatocytes were radially distributed around the central vein, and the hepatic sinus was clearly visible. Liver sections from the model group had hepatocyte swelling, ballooning degeneration, different sizes of lipid droplets, and hepatic cord changes. Liver sections from the PH group had mild steatosis, small lipid droplets that were visible in some cells, and disorderly arranged hepatic cords. The liver sections from the CHM group had diffused hepatocyte steatosis with different degrees of steatosis that were lower compared to that of the model group (Figure 1).

ORO staining

The liver tissues from control animals were histopathologically normal. Diffused and granular lipid droplets were observed in the liver sections from the model group by ORO staining. Compared to rats in the model group, rats in the PH and CHM group had markedly reduced lipid droplet accumulation in their hepatocytes (Table 3 and Figure 2).

Collagen fiber content

There was no increase in collagen tissue fibers in the model, PH, and CHM groups when compared to the control group. Masson's trichrome staining of liver sections demonstrated the absence of liver fibrosis in NAFLD rats (Figure 3).

Effect of CHM on blood lipid levels

Compared to the normal control group, there was a significant increase in TC, TG, and LDL levels and a significant decrease in HDL levels in the model group ($P < 0.01$). However, compared to the model group, the TC, TG, and LDL levels were significantly decreased, while HDL levels were significantly increased in both the PH and CHM groups ($P < 0.01$, $P < 0.05$) (Table 4).

Effect of CHM on TG level in liver tissue

Compared to the normal control group, there was a significant increase in TG level in liver tissue in the model, PH, and CHM groups ($P < 0.01$). Compared to the model group, the TG level in liver tissue was significantly decreased in both the PH and CHM groups ($P < 0.01$) (Table 5).

Blood glucose levels

Compared to the normal control group, blood glucose levels in the model group increased significantly ($P < 0.01$). However, compared to the model group, blood glucose levels were significantly decreased in the PH and CHM groups ($P < 0.01$) (Table 6).

Effect of CHM on serum AST and ALT levels

Compared to the normal control group, serum AST and ALT levels were significantly increased in the model group ($P < 0.01$). However, compared to the model group, serum AST and ALT levels were significantly decreased in the PH and CHM groups ($P < 0.01$, $P < 0.05$) (Table 7).

Effect of CHM on liver tissue TNF-α, TGF-β1, NF-kB, and TLR4 levels

Compared to the normal control group, TNF-α, TGF-β1, NF-kB, and TLR4 levels were significantly increased in the model group ($P < 0.01$). However, compared to the model group, TNF-α, TGF-β1, NF-kB, and TLR4 levels were significantly decreased in the PH and CHM groups ($P < 0.01$, $P < 0.05$) (Table 8).

Effect of CHM on intestinal sIgA levels

Table 2 Liver coefficient (mean ± SD)

Group	*n*	Liver weight (g)	Body weight (g)	Liver coefficient (%)
Control	8	11.56 ± 2.42	540.43 ± 90.11	2.13 ± 0.11
Model	8	15.35 ± 2.08	451.09 ± 43.89	3.41 ± 0.43[b]
PH	8	10.81 ± 1.00	456.48 ± 42.41	2.37 ± 0.08[d]
CHM	8	13.21 ± 2.18	437.81 ± 47.93	3.01 ± 0.21[bdf]

[a]$P < 0.05$,
[b]$P < 0.01$ *vs* control group; [c]$P < 0.05$,
[d]$P < 0.01$ *vs* model group; [e]$P < 0.05$,
[f]$P < 0.01$ *vs* PH group. PH: Pioglitazone hydrochloride; CHM: Chinese herbal medicine.

Compared to the control group, intestinal sIgA levels were significantly decreased in the model group, PH group, and CHM group ($P < 0.01$, $P < 0.05$). However, compared to the control group, intestinal sIgA levels were significantly increased in the PH group ($P < 0.01$). Compared to the PH group, intestinal sIgA levels were significantly decreased in the CHM group ($P < 0.01$) (Table 9).

Intestinal occludin and ZO-1 protein levels

Compared to the control group, occludin and ZO-1 protein expression was significantly decreased in the model and CHM groups ($P < 0.01$). Compared to the model group, occludin and ZO-1 protein expression was significantly increased in the PH group ($P < 0.01$). Compared to the PH group, occludin and ZO-1 protein levels were significantly decreased in the CHM group ($P < 0.01$) (Table 10 and Figure 4).

Intestinal occludin and ZO-1 mRNA levels

Compared to the control group, occluding and ZO-1 mRNA expression was significantly decreased in the model and CHM groups ($P < 0.01$). Compared to the model group, occludin and ZO-1 mRNA expression levels were significantly increased in the PH group ($P < 0.01$). Compared to the PH group, occludin and ZO-1 mRNA expression levels were significantly decreased in the CHM group ($P < 0.01$, $P < 0.05$) (Table 11).

Plasma endotoxin levels

Compared to the control group, plasma endotoxin level was significantly increased in the model group ($P < 0.01$). Compared to the model group, plasma endotoxin levels were significantly decreased in the PH and CHM groups ($P < 0.01$) (Table 12).

DISCUSSION

Epidemiological studies have demonstrated that liver disease remains a major health concern worldwide. NAFLD and its subtype non-alcoholic steatohepatitis affect approximately 35% of the worldwide population. Half of all NAFLD deaths are due to cardiovascular disease and malignancy. Despite recent medical breakthroughs, modern medicine still lacks an efficacious hepato-protective therapy with few side effects[14]. Based on the characteristics of traditional Chinese medicine and good efficacy of CHM, we aimed to investigate the regulatory effect of a CHM formula (Chinese thorowax root, scutellaria root, and white peony root) on NAFLD.

Previous studies have found that saikosaponin contained in Chinese thorowax root is effective in reducing experimental liver damage, reducing transaminase levels, and lowering cholesterol, TG, and phospholipid levels. Scutellaria root contains baicalein, flavonoid II, and baicalin. Experimental studies have demonstrated that oral flavonoid II could significantly reduce serum cholesterol levels in experimental hyperlipidemic rats fed a high fat diet. In addition, baicalein and baicalin are effective in reducing TG levels. White peony has also been demonstrated to have a protective role on the liver and detoxification functions.

Dyslipidemia is one of the hallmarks of metabolic syndrome and has been shown to be strongly associated with NAFLD. Dyslipidemia associated with NAFLD is typically characterized by elevated levels of circulating TG and LDL-C, as well as decreased HDL-C levels. In addition, previous studies have demonstrated that dietary fructose significantly increases TG plasma levels within a relatively short period of time in both rats and mice[15]. In the present study, compared to rats in the normal group, the liver coefficient and levels of serum TG, TC, LDL, fasting blood glucose,

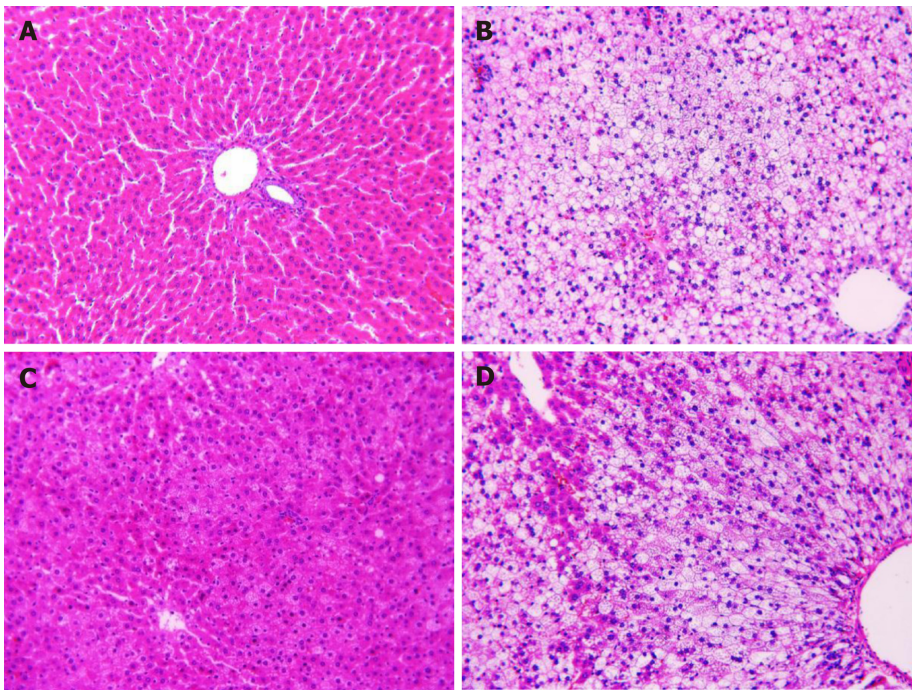

Figure 1 Histopathological examination by hematoxylin-eosin staining (200×). A: Control group; B: Model group; C: Pioglitazone hydrochloride group; D: Chinese herbal medicine group.

AST and ALT, and TG in liver tissue were significantly increased, while the levels of HDL were markedly decreased in NAFLD model rats. Administration of the CHM formula (Chinese thorowax root, scutellaria root, and white peony root) was able to decrease the levels of serum TG, TC, LDL, fasting blood sugar, AST, ALT, and TG in liver tissue while increasing serum HDL levels. Morphological changes in the liver correlated with liver function. Hepatocyte swelling, balloon degeneration, lipid droplet size, and hepatic cord disorders were observed in rats in the NAFLD model group. Treatment with CHM decoction (Chinese thorowax root, scutellaria root, and white peony root) reduced these histopathological changes in rats induced with a high-fat high-fructose diet. These results suggested that the CHM decoction (Chinese thorowax root, scutellaria root, and white peony root) is efficacious in protecting the liver and lowering lipid levels. CHM may function by "soothing the liver and gallbladder".

Previous studies have shown that high-fat diet-induced NAFLD in rats had higher levels of oxidative stress factors, lipid peroxidation products, and pro-inflammatory cytokines[14]. TNF-α is a cell signaling protein that is involved in systemic inflammation and has been associated with insulin resistance. TNF-α is produced predominantly by Kupffer cells (monocyte macrophage lineage) in the liver. Kupffer cells produce several cytokines that modulate TNF-α levels[1,16]. Rats in the Chinese herbal formula treatment group had significantly lower levels of TNF-α compared to rats in the NAFLD model group. These results showed that the CHM decoction (Chinese thorowax root, scutellaria root, and white peony root) is efficacious in reducing TNF-α levels and inflammation in rats fed a high-fat-high-fructose diet.

TGF-β is a proinflammatory cytokine whose role in the kidney, liver, and lungs, and in cardiac fibrosis is well documented[17]. During liver fibrosis, TGF-β1 levels are markedly increased in stellate cells. TGF-β2 is primarily expressed in Kupffer cells, followed by stellate and endothelial cells. A previous study using a murine steatohepatitis model observed that severe necro-inflammation and fibrosis were accompanied by an increase in TGF-β expression[18]. Results from our study demonstrated that TGF-β was significantly increased in rats in the NAFLD model group compared to the control group. Treatment with PH and CHM decoction (Chinese thorowax root, scutellaria root, and white peony root) significantly attenuated TGF-β levels.

In murine models of high-fat diet-induced steatosis, increased NF-kB activity has been associated with elevated levels of hepatic inflammatory cytokines, including TNF-α and activation of Kupffer cells. NF-kB modulates DNA transcription involved in inflammatory processes, infection, and apoptotic processes. NF-kB dysfunction may lead to inflammatory and autoimmune diseases. A number of studies have

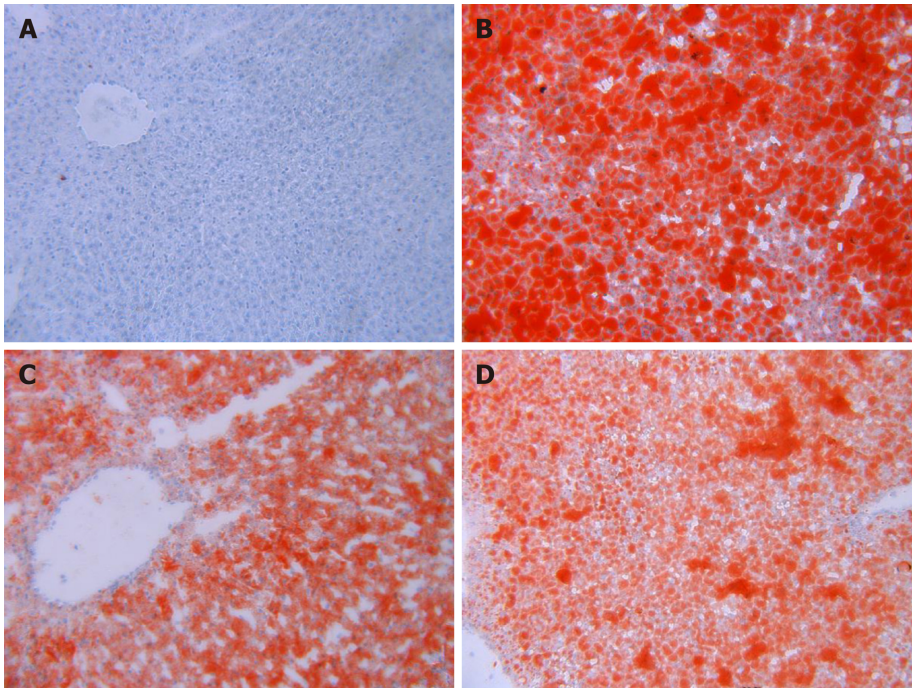

Figure 2 Histopathological examination by Oil red O staining (200×). A: Control group; B: Model group; C: Pioglitazone hydrochloride group; D: Chinese herbal medicine group.

demonstrated that NF-kB activation contributes to the pathogenesis of NAFLD/NASH and the development of HCC[8]. Results from our study showed that NF-kB levels were significantly increased in rats in the NAFLD model group compared to the control group, while the CHM decoction significantly reduced NF-kB levels in rats in the NAFLD model group.

Lipopolysaccharide-binding protein (LBP) is an acute-phase protein that is derived from the liver. LBP binds to the lipid A portion of lipopolysaccharide and interacts with TLR-4 to induce the downstream signaling pathways of the innate immune system, such as NF-kB and activator protein 1, the major transcription factors involved in inflammation. Activation of the TLR-4 signaling pathway by lipopolysaccharide and LBP complex has been shown to lead to NAFLD progression in animal models from simple fatty liver to steatohepatitis[19]. In this study, compared to the normal group, TLR4 levels were increased significantly in the NAFLD model group, while treatment with the Chinese herbal formula decreased TLR4 levels compared to the model group.

The "intestinal-liver" axis is closely associated with the occurrence of NAFLD. During NAFLD, the intestinal mucosal mechanical barrier is compromised, leading to increased barrier permeability[20]. The important structures that make up the intestinal mechanical barrier is the tight junctions around the apical side of the mucosal epithelial cells and are mainly composed of occludin, claudin, junction adhesion molecules (JAMs), and ZOs. The main function of the intestinal mucosal immune barrier is to produce a local immune response after antigenic stimulation and protect the body from damage by neutralizing the antigen. These involve the immune organs, immune cells and secretion of sIgA induced by cytokines[21]. When the mechanical barrier of the intestinal mucosa is severely damaged, the bacteria are translocated, endotoxin is circulated into the liver through the portal system, and liver Kupffer cells are activated, thereby releasing a series of inflammatory factors and leading to hepatocyte inflammatory reaction and fibrosis[22]. In this study, we investigated the effects of traditional Chinese medicine on liver tissue-associated inflammatory factors and tight junction proteins on the intestinal mucosa and investigated whether the CHM decoction could play a role in the treatment of NAFLD by regulating the "intestinal-liver" axis. Our results demonstrated that compared to the control group, intestinal sIgA levels were significantly decreased in the model, PH, and CHM groups, while intestinal sIgA levels in the PH group were significantly higher compared to the model and CHM groups. Compared with the control group, the gene and protein expression of occludin and ZO-1 decreased significantly in the model group and CHM group, while it was significantly higher in the PH group compared to the model and CHM groups. Compared to the control group, plasma endotoxin

Table 3 Hepatocyte lipid droplet cavity area ratio (mean ± SD)

Group	*n*	Area ratio (%)
Control	5	0.00 ± 0.00
Model	5	72.15 ± 1.72
PH	5	31.01 ± 7.53[a]
CHM	5	48.69 ± 5.85[a]

[a]$P < 0.05$ *vs* model group. PH: Pioglitazone hydrochloride; CHM: Chinese herbal medicine.

level was significantly increased in the model group, while it was significantly decreased in the PH and CHM groups compared to the model group. Our results indicated that the intestinal barrier and endotoxin affect the occurrence of NAFLD, and there is a certain correlation between the intestine and liver. The CHM decoction can affect both the liver and intestine, while the effect of the CHM decoction on the liver is superior to that on the intestine.

In conclusion, the CHM decoction (thorowax root, scutellaria root, and white peony root) has a good effect in regulating lipid metabolism and liver function, which suggests its ability to affect the liver. To a certain extent, it also has a regulatory effect on the intestinal mucosal barrier. Our study demonstrates that the mechanism of "soothing the liver" by DCHD for the treatment of NAFLD is mainly through the effect on the liver.

Table 4 Effect of Chinese herbal medicine on blood lipid levels (mean ± SD, *n* = 8)

Group	TG (mmol/L)	TC (mmol/L)	LDL (mmol/L)	HDL (mmol/L)
Control	0.48 ± 0.20	1.67 ± 0.26	0.24 ± 0.08	0.95 ± 0.10
Model	0.83 ± 0.12[b]	3.05 ± 0.67[b]	1.38 ± 0.33[b]	0.54 ± 0.05[b]
PH	0.50 ± 0.08[d]	1.69 ± 0.29[d]	0.76 ± 0.28[bd]	0.61 ± 0.05[bc]
CHM	0.53 ± 0.12[d]	2.04 ± 0.37[d]	0.84 ± 0.16[bd]	0.72 ± 0.05[bdf]

[b]$P < 0.01$ *vs* control group;
[c]$P < 0.05$,
[d]$P < 0.01$ *vs* model group;
[f]$P < 0.01$ *vs* PH group. PH: Pioglitazone hydrochloride; CHM: Chinese herbal medicine; TC: Total cholesterol; TG: Triglyceride; HDL: High density lipoprotein; LDL: Low density lipoprotein.

Table 5 Effect of Chinese herbal medicine on triglyceride levels in liver tissue (mean ± SD)

Group	*n*	TG (mmol/L)
Control	8	1.37 ± 0.67
Model	8	4.81 ± 0.72[b]
PH	8	3.41 ± 0.43[bd]
CHM	8	3.41 ± 0.66[bd]

[b]$P < 0.01$ *v* control group;
[d]$P < 0.01$ *vs* model group. TG: Triglyceride; PH: Pioglitazone hydrochloride; CHM: Chinese herbal medicine.

Table 6 Effect of Chinese herbal medicine on blood glucose levels (mean ± SD)

Group	*n*	Blood sugar (mmol/L)
Control	8	4.09 ± 0.26
Model	8	4.90 ± 0.33[b]
PH	8	4.11 ± 0.20[d]
CHM	8	4.28 ± 0.21[d]

[b]$P < 0.01$ *vs* control group;
[d]$P < 0.01$ *vs* model group. PH: Pioglitazone hydrochloride; CHM: Chinese herbal medicine.

Table 7 Effect of Chinese herbal medicine on serum liver function markers (mean ± SD)

Group	*n*	AST (U/L)	ALT (U/L)
Control	8	118.73 ± 13.71	39.91 ± 6.50
Model	8	238.13 ± 39.38[b]	59.28 ± 9.69[b]
PH	8	137.94 ± 5.83[d]	48.84 ± 8.28[ac]
CHM	8	148.19 ± 20.59[ad]	49.36 ± 5.13[ac]

[a]$P < 0.05$,
[b]$P < 0.01$ *vs* control group;
[c]$P < 0.05$,
[d]$P < 0.01$ *vs* model group. PH: Pioglitazone hydrochloride; CHM: Chinese herbal medicine; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase.

Table 8 Effect of Chinese herbal medicine on liver tissue TNF-α, TGF-β1, NF-kB, and TLR4 levels (mean ± SD)

Group	*n*	TNF-α (ng/mg)	TGF-β1 (ng/mg)	NF-kB (ng/mg)	TLR4 (ng/mg)
Control	8	5.35 ± 0.80	2.21 ± 0.43	10.87 ± 2.45	21.32 ± 1.87
Model	8	7.22 ± 0.81[b]	3.01 ± 0.22[b]	15.85 ± 2.50[b]	30.28 ± 4.38[b]
PH	8	5.48 ± 0.38[d]	2.24 ± 0.26[d]	12.15 ± 3.13[c]	22.56 ± 3.02[d]
CHM	8	6.01 ± 0.97[d]	2.29 ± 0.40[d]	12.57 ± 3.30	23.88 ± 3.21[d]

[b]$P < 0.01$ *vs* control group;
[c]$P < 0.05$,
[d]$P < 0.01$ *vs* model group. TGF-β1: Transforming growth factor-β1; TNF-α: Tumor necrosis factor-α; TLR4: Toll-like receptor-4; NF-kB: Nuclear factor-kappa B; PH: Pioglitazone hydrochloride; CHM: Chinese herbal medicine.

Table 9 Intestinal sIgA levels (mean ± SD)

Group	*n*	sIgA (μg/mg)
Control	8	19.80 ± 2.25
Model	8	11.39 ± 1.81[b]
PH	8	17.06 ± 2.92[ad]
CHM	8	12.78 ± 2.36[bf]

[a]$P < 0.05$,
[b]$P < 0.01$ *vs* control group;
[d]$P < 0.01$ *vs* model group; [ef]$P < 0.01$ *vs* PH group. sIgA: Secreted immunoglobulin A; PH: Pioglitazone hydrochloride; CHM: Chinese herbal medicine.

Table 10 Occludin and zonula occludens-1 protein levels (mean ± SD)

Group	*n*	Occludin	ZO-1
Control	7	1.12 ± 0.06	1.41 ± 0.03
Model	7	0.66 ± 0.07[b]	0.82 ± 0.09[b]
PH	7	1.04 ± 0.09[d]	1.32 ± 0.04[d]
CHM	7	0.68 ± 0.05[bf]	0.84 ± 0.12[bf]

[b]$P < 0.01$ *vs* control group;
[d]$P < 0.01$ *vs* model group;
[f]$P < 0.01$ *vs* PH group. ZO-1: Zonula occludens-1; PH: Pioglitazone hydrochloride; CHM: Chinese herbal medicine.

Table 11 Occludin and zonula occludens-1 mRNA expression levels (mean ± SD)

Group	*n*	Occludin mRNA	ZO-1 mRNA
Control	6	1.40 ± 0.19	1.31 ± 0.29
Model	6	0.60 ± 0.16[b]	0.60 ± 0.11[b]
PH	6	1.15 ± 0.24[ad]	1.15 ± 0.23[d]
CHM	6	0.70 ± 0.13[bf]	0.79 ± 0.31[be]

[a]$P < 0.05$,
[b]$P < 0.01$ *vs* control group;
[d]$P < 0.01$ *vs* model group;
[e]$P < 0.05$,
[f]$P < 0.01$ *vs* PH group. ZO-1: Zonula occludens-1; PH: Pioglitazone hydrochloride; CHM: Chinese herbal medicine.

Table 12 Levels of plasma endotoxin (mean ± SD)

Group	*n*	Endotoxin (%)
Control	8	0.028 ± 0.013
Model	8	0.160 ± 0.057[b]
PH	8	0.045 ± 0.012[d]
CHM	8	0.056 ± 0.018[d]

[b]$P < 0.01$ *vs* control group;
[d]$P < 0.01$ *vs* model group. PH: Pioglitazone hydrochloride; CHM: Chinese herbal medicine.

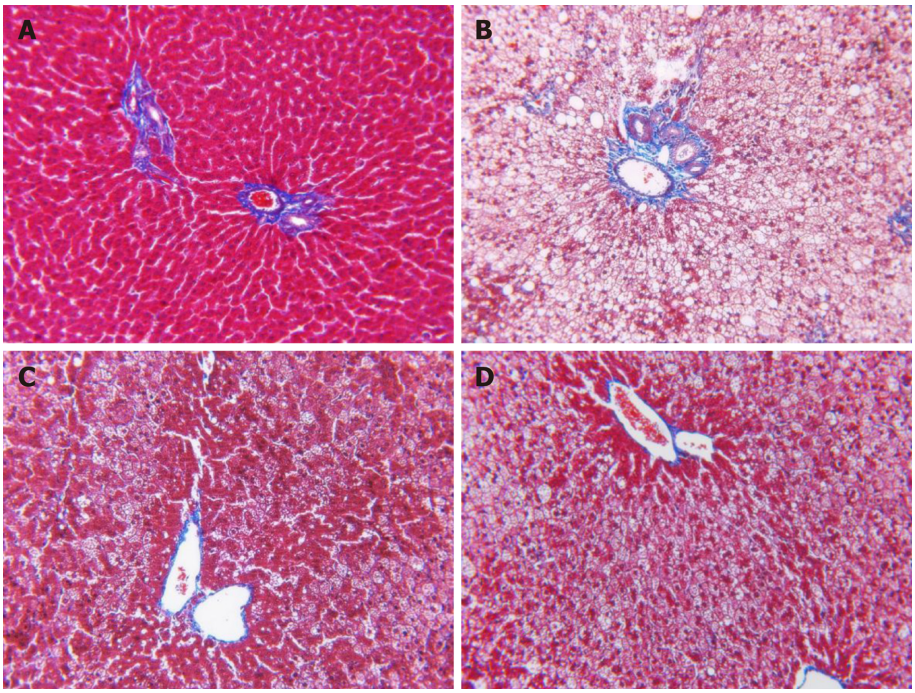

Figure 3 Masson's trichrome staining of liver tissue sections (200×). A: Control group; B: Model group; C: Pioglitazone hydrochloride group; D: Chinese herbal medicine group.

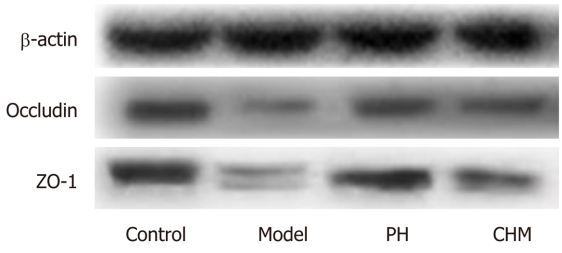

Figure 4 Occludin and Zonula occludens-1 protein expression in intestine determined by Western blot using β-actin as a reference protein.

ARTICLE HIGHLIGHTS

Research background

Non-alcoholic fatty liver disease (NAFLD) has become one of the most major causes of chronic liver disease and affects both adults and children. There are currently no effective treatment options for NAFLD, with the exemption of lifestyle changes. The Chinese herbal medicine (CHM) Dachaihu decoction (DCHD) has been proved to treat NAFLD with good efficacy in previous studies.

Research motivation

Based on the TCM principle of formula formation and good efficacy of CHM, we divided DCHD

into soothing liver part, invigorating spleen part, and dredging intestine part. Marshall officially proposed the concept of "intestinal-hepatic axis", which systematically explains the interactions between intestine and liver. We hypothesized that the effect of CHM on NAFLD is achieved by regulating the liver and intestine.

Research objectives

We aimed to investigate the possible effect of a CHM formula on NAFLD in a rat model, which will provide more evidence for the therapeutic effect of CHM on NAFLD in the future.

Research methods

Sixty rats were randomly divided into four groups: Control, model, PH, and CHM (Chinese thorowax root, scutellaria root, and white peony root) groups. An NAFLD rat model was established using a high-fat high-fructose diet for 16 wk. From the 13th week, rats in PH group and CHM group were administered with PH solution and a decoction of Chinese thorowax, scutellaria, and white peony root, respectively. Rats in the control group and model group were administered with an equal volume of distilled water. At the end of the study, blood was collected *via* the abdominal aorta. Liver tissues were harvested and any morphological changes were observed by hematoxylin-eosin (HE) staining, Oil red O staining, and Masson staining. In addition, blood lipids, liver function markers, and TG in liver tissues were analyzed. The levels of transforming growth factor-β1 (TGF-β1), tumor necrosis factor-α (TNF-α), Toll-like receptor-4 (TLR4), and nuclear factor-kappa B (NF-κB) in liver tissues and sIgA in intestinal tissues were analyzed by ELISA, and protein and mRNA expression of occludin and ZO-1 in the intestine were measured using Western blot and reverse transcription-quantitative polymerase chain reaction, respectively. The endotoxin level in plasma was detected by endpoint chromogenic assay. SPSS17.0 software package (IBM, Armonk, NY, United States) was used for statistical analysis.

Research results

Compared to the normal control group, the liver coefficient, serum TG, TC, LDL, AST, and ALT, blood glucose, the levels of TG, TNF-α, TGF-β, NF-kB, and TLR4 in liver tissues, and plasma endotoxin increased significantly in the model group, while serum HDL, intestinal sIgA, and protein and mRNA expression of occludin and ZO-1 decreased significantly in the model group ($P < 0.01$). PH and CHM attenuated the elevated liver coefficient, serum TG, TC, LDL, AST, and ALT, blood glucose, the levels of TG, TNF-α, TGF-β, NF-kB, and TLR4 in liver tissues, and plasma endotoxin, and increased serum HDL levels compared to the model group ($P < 0.01$). Intestinal sIgA and the protein and mRNA expression of intestinal occludin and ZO-1 were significantly increased in the PH group compared to the model and CHM groups ($P < 0.01$).

Research conclusions

In this study, the CHM decoction (Chinese thorowax root, scutellaria root, and white peony root) is beneficial in regulating lipid metabolism and liver function, which indicates that they have a good effect on the liver. To a certain extent, the CHM decoction can affect both the liver and intestine, while the effect of the CHM decoction on the liver is superior to that on the intestine.

Research perspectives

This study demonstrated that the mechanism of the soothing liver part of DCHD for the treatment of NAFLD was mainly *via* the regulation of the liver. The Chinese formula does have its special formation principle. Different CHMs in the same formula are effective on different syndromes. This study proposed formula division and tried to find the relationship between Chinses herbs and internal organs (liver and intestine), which will provide more experimental evidence for the therapeutic effect of CHM on NAFLD in the future. This study preliminary applied the liver-intestine axis to explain the relationship between Chinese formula and NAFLD.

REFERENCES

1 **Jiang W**, Guo MH, Hai X. Hepatoprotective and antioxidant effects of lycopene on non-alcoholic fatty liver disease in rat. *World J Gastroenterol* 2016; **22**: 10180-10188 [PMID: 28028366 DOI: 10.3748/wjg.v22.i46.10180]

2 **Tan DY**, Shi HY, Li CP, Zhong XL, Kang M. Effect of nuclear factor-κB and angiotensin II receptor type 1 on the pathogenesis of rat non-alcoholic fatty liver disease. *World J Gastroenterol* 2015; **21**: 5877-5883 [PMID: 26019451 DOI: 10.3748/wjg.v21.i19.5877]

3 **Vespasiani-Gentilucci U**, Gallo P, Dell'Unto C, Volpentesta M, Antonelli-Incalzi R, Picardi A. Promoting genetics in non-alcoholic fatty liver disease: Combined risk score through polymorphisms and clinical variables. *World J Gastroenterol* 2018; **24**: 4835-4845 [PMID: 30487694 DOI: 10.3748/wjg.v24.i43.4835]

4 **Kapravelou G**, Martínez R, Nebot E, López-Jurado M, Aranda P, Arrebola F, Cantarero S, Galisteo M, Porres JM. The Combined Intervention with Germinated Vigna radiata and Aerobic Interval Training Protocol Is an Effective Strategy for the Treatment of Non-Alcoholic Fatty Liver Disease (NAFLD) and Other Alterations Related to the Metabolic Syndrome in Zucker Rats. *Nutrients* 2017; 9 [PMID: 28753963 DOI: 10.3390/nu9070774]

5 **Christensen CU**, Glavind E, Thomsen KL, Kim YO, Heebøll S, Schuppan D, Hamilton-Dutoit S, Würtz Heegaard C, Grønbæk H. Niemann-Pick type C2 protein supplementation in experimental non-alcoholic fatty liver disease. *PLoS One* 2018; **13**: e0192728 [PMID: 29522534 DOI: 10.1371/journal.pone.0192728]

6 **Nie J**, Li CP, Li JH, Chen X, Zhong X. Analysis of nonalcoholic fatty liver disease microRNA expression spectra in rat liver tissues. *Mol Med Rep* 2018; **18**: 2669-2680 [PMID: 30015905 DOI: 10.3892/mmr.2018.9268]

7 **Veena J**, Muragundla A, Sidgiddi S, Subramaniam S. Non-alcoholic fatty liver disease: need for a balanced nutritional source. *Br J Nutr* 2014; **112**: 1858-1872 [PMID: 25274101 DOI: 10.1017/S0007114514002591]

8 **Guo Y**, Li JX, Mao TY, Zhao WH, Liu LJ, Wang YL. Targeting Sirt1 in a rat model of high-fat diet-induced non-alcoholic fatty liver disease: Comparison of Gegen Qinlian decoction and resveratrol. *Exp Ther Med* 2017; **14**: 4279-4287 [PMID: 29104641 DOI: 10.3892/etm.2017.5076]

9 **Gao XM**. "Chinese herbal medicinal," People's Medical Publishing House, Beijing, China, 2nd edition, 2013

10 **Fan YH**, Miao BQ. Treatment of 63 Cases of Nonalcoholic Fatty Liver disease with Dachaihu Decoction. *Mongol J Traditional Chinese Medicine* 2014; **33**: 9

11 **Lin LL**, Li LL. Clinical Observation on Treatment of Nonalcoholic Fatty Liver with Combination of Traditional Chinese Medicine and Western Medicine. *J Practical Traditional Chinese Medicine* 2017; **33**: 1069-1070

12 **Huang ZL**, Huang XJ, Huang XL, Liu FZ, Hu NN, Huang GX. Effects of diacerein on inflammatory cytokines and adipose metabolism as well as the expression of chemerin in adipose tissue of type 2 diabetic rats. *J Xi'an Jiaotong University (Medical Sciences)* 2017; **38**: 693-698

13 **Chen Q**. "Research methods in pharmacology of Chinese materia medica" Beijing, People's Medical Publishing House, Beijing, China, 2nd edition, 2006

14 **Ou-Yang Q**, Xuan CX, Wang X, Luo HQ, Liu JE, Wang LL, Li TT, Chen YP, Liu J. 3-Acetyl-oleanolic acid ameliorates non-alcoholic fatty liver disease in high fat diet-treated rats by activating AMPK-related pathways. *Acta Pharmacol Sin* 2018; **39**: 1284-1293 [PMID: 29345253 DOI: 10.1038/aps.2017.142]

15 **Jensen VS**, Hvid H, Damgaard J, Nygaard H, Ingvorsen C, Wulff EM, Lykkesfeldt J, Fledelius C. Dietary fat stimulates development of NAFLD more potently than dietary fructose in Sprague-Dawley rats. *Diabetol Metab Syndr* 2018; **10**: 4 [PMID: 29410708 DOI: 10.1186/s13098-018-0307-8]

16 **Tsai CC**, Lin YJ, Yu HR, Sheen JM, Tain YL, Huang LT, Tiao MM. Melatonin alleviates liver steatosis induced by prenatal dexamethasone exposure and postnatal high-fat diet. *Exp Ther Med* 2018; **16**: 917-924 [PMID: 30112044 DOI: 10.3892/etm.2018.6256]

17 **Soofi A**, Wolf KI, Emont MP, Qi N, Martinez-Santibanez G, Grimley E, Ostwani W, Dressler GR. The kielin/chordin-like protein (KCP) attenuates high-fat diet-induced obesity and metabolic syndrome in mice. *J Biol Chem* 2017; **292**: 9051-9062 [PMID: 28424263 DOI: 10.1074/jbc.M116.771428]

18 **Song L**, Qu D, Zhang Q, Jiang J, Zhou H, Jiang R, Li Y, Zhang Y, Yan H. Phytosterol esters attenuate hepatic steatosis in rats with non-alcoholic fatty liver disease rats fed a high-fat diet. *Sci Rep* 2017; **7**: 41604 [PMID: 28169366 DOI: 10.1038/srep41604]

19 **Nien HC**, Sheu JC, Chi YC, Chen CL, Kao JH, Yang WS. One-year weight management lowers lipopolysaccharide-binding protein and its implication in metainflammation and liver fibrosis. *PLoS One* 2018; **13**: e0207882 [PMID: 30458048 DOI: 10.1371/journal.pone.0207882]

20 **Xin D**, Zong-Shun L, Bang-Mao W, Lu Z. Expression of intestinal tight junction proteins in patients with non-alcoholic fatty liver disease. *Hepatogastroenterology* 2014; **61**: 136-140 [PMID: 24895809 DOI: 10.5754/hge12760]

21 **Ito H**, Takemura N, Sonoyama K, Kawagishi H, Topping DL, Conlon MA, Morita T. Degree of polymerization of inulin-type fructans differentially affects number of lactic acid bacteria, intestinal immune functions, and immunoglobulin A secretion in the rat cecum. *J Agric Food Chem* 2011; **59**: 5771-5778 [PMID: 21506616 DOI: 10.1021/jf200859z]

22 **Miele L**, Marrone G, Lauritano C, Cefalo C, Gasbarrini A, Day C, Grieco A. Gut-liver axis and microbiota in NAFLD: insight pathophysiology for novel therapeutic target. *Curr Pharm Des* 2013; **19**: 5314-5324 [PMID: 23432669 DOI: 10.2174/13816128113192900011]

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ORIGINAL ARTICLE

Basic Study

Allyl isothiocyanate ameliorates lipid accumulation and inflammation in nonalcoholic fatty liver disease *via* the Sirt1/AMPK and NF-κB signaling pathways

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Author contributions: Li CX and Gao JG designed and performed the study; Wan XY, Chen Y, Xu CF and Feng ZM performed the research; Lin YM and Yu CH analyzed the data; Ma H and Xu P provided guidance during revision; Li YM supervised the study and provided consultation during the entire study.

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Abstract

BACKGROUND

Allyl isothiocyanate (AITC), a classic anti-inflammatory and antitumorigenic agent, was recently identified as a potential treatment for obesity and insulin resistance. However, little is known about its direct impact on the liver.

AIM

To investigate the effect and underlying mechanism of AITC in nonalcoholic fatty liver disease (commonly referred to as NAFLD).

METHODS

To establish a mouse and cellular model of NAFLD, C57BL/6 mice were fed a high fat diet (HFD) for 8 wk, and AML-12 cells were treated with 200 μM palmitate acid for 24 h. For AITC treatment, mice were administered AITC (100 mg/kg/d) orally and AML-12 cells were treated with AITC (20 μmol/L).

RESULTS

AITC significantly ameliorated HFD-induced weight gain, hepatic lipid accumulation and inflammation *in vivo*. Furthermore, serum alanine aminotransferase and aspartate aminotransferase levels were markedly reduced in AITC-treated mice. Mechanistically, AITC significantly downregulated the protein levels of sterol regulatory elementbinding protein 1 (SREBP1) and its lipogenesis target genes and upregulated the levels of proteins involved in fatty

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ARRIVE guidelines statement: The authors have read the ARRIVE guidelines and prepared and revised the manuscript according to the ARRIVE guidelines.

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acid β-oxidation, as well as the upstream mediators Sirtuin 1 (Sirt1) and AMP-activated protein kinase α (AMPKα), in the livers of HFD-fed mice. AITC also attenuated the nuclear factor kappa B (NF-κB) signaling pathway. Consistently, AITC relieved palmitate acid-induced lipid accumulation and inflammation in AML-12 cells *in vitro* through the Sirt1/AMPK and NF-κB signaling pathways. Importantly, further studies showed that the curative effect of AITC on lipid accumulation was abolished by siRNA-mediated knockdown of either Sirt1 or AMPKα in AML-12 cells.

CONCLUSION
AITC significantly ameliorates hepatic steatosis and inflammation by activating the Sirt1/AMPK pathway and inhibiting the NF-κB pathway. Therefore, AITC is a potential therapeutic agent for NAFLD.

Key words: Allyl isothiocyanate; Nonalcoholic fatty liver disease; Hepatic steatosis; Liver inflammation

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Core tip: Nonalcoholic fatty liver disease (NAFLD) is rapidly prevalent as a remarkable problem worldwide. We aimed to investigate the therapeutic role of allyl isothiocyanate (AITC) in lipid accumulation and inflammation during NAFLD development in mice fed a high fat diet and AML-12 cells treated with palmitate acid. Our study for the first time demonstrates that AITC ameliorates hepatic steatosis and inflammation by activating the Sirt1/AMPK and IKK/NF-κB signaling pathway. This study reveals role for AITC as a potential therapeutic agent for NAFLD.

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INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is currently one of the most epidemic chronic liver diseases worldwide and has become an enormous clinical and economic burden. NAFLD affects approximately one-fourth of adults globally[1]. NAFLD represents a wide spectrum of disease stages, ranging from simple steatosis to nonalcoholic steatohepatitis (commonly known as NASH), which is characterized by hepatocellular injury and inflammation and may eventually progress to cirrhosis and hepatocellular carcinoma[2]. Furthermore, NAFLD is a strong risk factor for type 2 diabetes, atherosclerosis, cardiovascular disease and chronic kidney disease[3-5]. However, its pathogenesis remains unclear, and current therapeutic options are relatively limited. No treatments for NAFLD other than lifestyle modifications leading to weight loss and increased physical activity are currently approved[6]. Therefore, there is an urgent need to develop effective medical treatments for NAFLD.

Hepatic steatosis, characterized by excessive triglyceride (TG) accumulation in hepatocytes, is strongly associated with chronic hepatic inflammation and insulin resistance[7]. Furthermore, the IκB kinase (IKK)/nuclear factor kappa B (NF-κB) signaling pathway plays a crucial role in the development of metabolic disorders, including NAFLD, and especially in hepatic inflammation[8-10].

Sirtuin 1 (Sirt1) is a highly conserved nicotinamide adenine dinucleotide-dependent protein deacetylase that regulates a wide variety of biological functions in mammals, including lipid metabolism and energy homeostasis[11,12]. AMP-activated protein kinase (AMPK) functions as an energy switch that controls several cellular processes, such as lipid metabolism, by inhibiting hepatic lipogenesis and stimulating fatty acid oxidation[13]. Previous studies revealed that Sirt1 is an important regulator of hepatic lipogenesis and fatty acid oxidation through multiple nutrient sensors, including sterol regulatory elementbinding protein 1 (SREBP1), peroxisome proliferator-

activated receptor gamma coactivator1α (PGC1α) and peroxisome proliferator-activated receptor α (PPARα)[14-16]. Furthermore, Sirt1 was shown to regulate AMPK activation in NAFLD, resulting in enhanced lipolysis and β-oxidation, as well as ameliorated hepatic steatosis[17-19].

Allyl isothiocyanate (AITC) is derived from its precursor sinigrin, which is present in many common cruciferous vegetables and is widely consumed by humans[20]. Myrosinase in the intestinal microflora catalyzes the hydrolysis of sinigrin to AITC in both humans and animals[20,21]. Previous studies have shown that AITC exhibits anti-inflammatory and anticancer activities[22-24]. Recently, AITC was identified as a potential novel treatment for diet-induced obesity and insulin resistance through its modulation of mitochondrial dysfunction[25]. Another study revealed that AITC can augment basal and epinephrine-induced lipolysis in adipocytes and intensify hydrolysis of TG in the blood serum of rats[26]. Moreover, a previous study showed AITC effectively inhibits adipogenic differentiation of 3T3-L1 preadipocytes and suppresses expression of genes up-regulated during adipogenesis[27]. However, little is known about its direct impact on the liver or its underlying mechanism.

Herein, we conducted both *in vivo* and *in vitro* experiments to explore the effect of AITC on NAFLD, focusing on its role in hepatic steatosis and inflammatory responses, and to elucidate its mechanism of action.

MATERIALS AND METHODS

Animal experiments

All experiments were conducted with approval of the First Affiliated Hospital of Zhejiang University Institutional Animal Care and Use Committee (Permit number: 2016-231). Six-week-old male C57BL/6 mice were purchased from B&K Laboratory Animal Corp., Ltd. (Shanghai, China). After acclimatization for 2 wk with free access to food and water, mice were fed a standard chow diet (SCD) or high fat diet (HFD) (60% fat-derived calories, 20% carbohydrate-derived calories, and 20% protein-derived calories; D12492, Research Diets, New Brunswick, NJ, United States). In general, mice were given SCD or HFD feeding for a total of 8 wk, and from the 5th wk, SCD-fed mice began to receive corn oil (control) (n = 10), and HFD-fed mice were randomly divided into two groups to receive 100 mg/kg/d AITC (99.7%; Sigma-Aldrich, St. Louis, MO, United States) (n = 10) or corn oil (n = 9) daily by gavage for an additional 4 wk while remaining on SCD or HFD.

Cell culture and treatments

The established immortalized AML-12 mouse hepatocyte cell line was purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). AML-12 cells were cultured in DMEM/F12 (1:1) medium supplemented with 10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, 0.1 μmol/L dexamethasone, and 1% insulin-transferrin-selenium Liquid Media Supplement (I3146; Sigma-Aldrich). To establish a cellular model of NAFLD, palmitate acid (PA) (Sigma-Aldrich) was dissolved in bovine serum albumin (Sangon Biotech, Shanghai, China), and then AML-12 cells were exposed to 200 μM PA for 24 h. To investigate the effect of AITC on lipid deposition *in vitro*, PA-stimulated AML-12 cells in serum-free conditions were treated with AITC (20 μmol/L) or dimethyl sulfoxide (commonly known as DMSO) (vehicle) for 24 h.

AML-12 cells were transfected with *Sirt1* small interfering RNA (siRNA) #1 (target sequence 5′-GATGAAGTTGACCTCCTCA-3′), *Sirt1* siRNA #2 (target sequence 5′-CCGATGGACTCCTCACTAA-3′), *Sirt1* siRNA #3 (target sequence 5′-GGTT GTTAATGAAGCTATA-3′), *AMPKa* siRNA #1 (target sequence 5′-GCAGAAGA TTCGGAGCCTT-3′), *AMPKa* siRNA #2 (target sequence 5′-GCACACCCTGGA TGAATTA-3′), *AMPKa* siRNA #3 (target sequence 5′-GCAGAAGTTTGTAGAGCAA-3′) or the corresponding scrambled control (RIBOBIO, Guangzhou, China) using Lipofectamine RNAiMAX (Invitrogen, Shanghai, China) according to the manufacturer's protocol. After 48 h, the cells were incubated in medium containing PA with or without AITC for an additional 24 h.

Hepatic and cellular TG assay

Hepatic and cellular TG contents were measured using a commercial kit (Applygen Technologies Inc., Beijing, China) according to the manufacturer's protocol.

Hematoxylin–eosin and oil red O staining

Mouse liver tissues were rapidly harvested, fixed in 10% formalin, embedded in paraffin, sectioned and stained with hematoxylin and eosin (commonly known as H&E) for histological examination. Frozen liver sections (8 μm) and cells in 6-well

plates were stained with oil red O (Sigma-Aldrich) to assess lipid accumulation.

Metabolic measurements

Plasma alanine aminotransferase (ALT), aspartate aminotransferase (AST), total cholesterol and uric acid levels were determined with a Hitachi 7600 autoanalyzer (Hitachi, Tokyo, Japan) according to the manufacturer's instructions.

Quantitative real-time PCR

Total mRNA was extracted from liver tissues or cultured cells using RNA plus (Takara, Dalian, China) and reverse transcribed into cDNA using a PrimeScript® RT reagent kit (Takara, Japan) according to the manufacturer's protocol. Real-time PCR was performed on an ABI Prism 7500 Sequence Detection System (Applied Biosystems, Foster City, CA, United States) using SYBR Green (Takara) to quantify PCR amplification. Relative mRNA expression levels of target genes were normalized to β-actin or GAPDH mRNA levels for each sample.

Western blot analysis

Liver tissue samples and cells were lysed using RIPA buffer (Applygen Technologies Inc.) supplemented with protease and phosphatase inhibitors (Sigma). Equal amounts of extracted proteins were separated by SDS-PAGE and transferred to PVDF membranes (Millipore, Inc., Darmstadt, Germany). Membranes were blocked with 5% nonfat milk in TBST and then incubated overnight at 4°C with the following primary antibodies: Anti-Sirt1 (8469), anti-AMPKα (2603), anti-phosphorylated (p) AMPKα (p-*AMPKa*; 2535), anti-NF-κB p65 (6956), anti-p-NF-κB p65 (3033), anti-p-IKK (2697), anti-*IKKa* (2682), anti-*IKKβ* (8943), anti-p- inhibitor of nuclear factor kappa B alpha (IκBα) (2859), anti-IκBα (4812), anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (2118), and β-actin (3700) (Cell Signaling Technology); and anti-PGC1α (ab54481), anti-PPARα (ab8934), anti-carnitine palmitoyltransferase 1 α (CPT1α) (ab128568), anti-SREBP1 (ab3259), anti-fatty acid synthase (FAS) (ab128856), anti-stearoyl coenzyme A desaturase 1 (SCD1) (ab19862), and anti-tubulin (ab6160) (Abcam, Cambridge, United Kingdom). Proteins levels were evaluated using an enhanced ECL kit (Lianke, Hangzhou, China).

Statistical analysis

The statistical methods of this study were reviewed by Hong Zhang from the Department of Statistics and Finance, School of Management, University of Science and Technology of China. All statistical analyses were performed with SPSS 22 (IBM, Chicago, IL, United States). Data are presented as the average ± S.D. Statistical analysis was carried out using Student's two-tailed *t*-test and one-way ANOVA with Tukey's post-test. *P*-values less than 0.05 indicated statistical significance.

RESULTS

AITC reduces body weight, ameliorates hepatic steatosis and attenuates liver injury in an HFD mouse model

Supplementary Figure 1 shows the chemical structure of AITC. To explore the effect of AITC on NAFLD, an HFD model, which is quite similar to but does not completely mirror human NAFLD, was adopted. HFD-fed mice exhibited higher body weight (Figure 1A and B) and liver weight (Figure 1D), more serious lipid accumulation (Figure 1C), much higher TG (Figure 1E), ALT (Figure 1F), AST (Figure 1G), cholesterol (Figure 1H) and uric acid (Figure 1I) levels compared to SCD-fed mice, showing characteristics of NAFLD *in vivo*. The mice exhibited a significant decrease in body weight upon 100 mg/kg/d AITC administration (Figure 1A and B) and a slight but nonsignificant decrease in liver weight (Figure 1D). Notably, hepatic steatosis was improved after AITC treatment, as demonstrated by decreased hepatic TG levels (Figure 1E) and reduced lipid accumulation (H&E and oil red O staining; Figure 1C). As shown in Figure 1F-I, AITC attenuated HFD-induced liver injury, as evidenced by markedly decreased serum ALT and AST levels. These findings demonstrate that AITC ameliorates body weight, hepatic steatosis and liver injury in HFD-fed mice.

AITC attenuates de novo lipogenesis and promotes fatty acid β-oxidation by activating the Sirt1/AMPK pathway in vivo

To investigate the mechanisms by which AITC ameliorates hepatic steatosis, the expression levels of hepatic lipid metabolism-related genes were measured. *SREBP1* is the central transcription factor that enhances the expression of genes required for hepatic fatty acid synthesis and TG synthesis[28]. As a result, AITC treatment *in vivo*

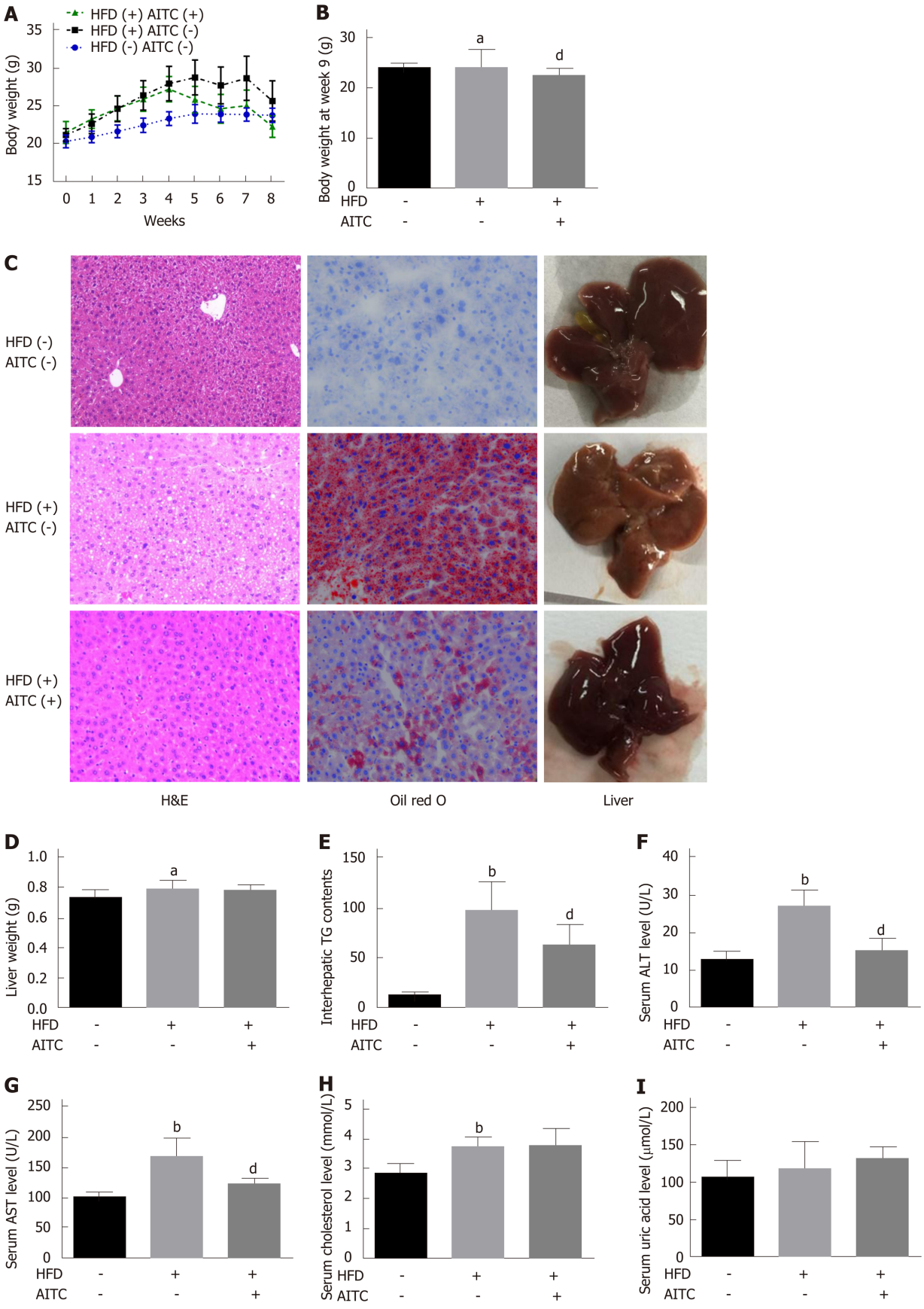

Figure 1 Allyl isothiocyanate reduces body weight, ameliorates hepatic steatosis and attenuates liver injury in high fat diet-fed mice. A: Body weight evaluated weekly; B: Body weight at week 8; C: Representative liver sections stained with hematoxylin and eosin (H&E) (left panel) or oil red O (middle panel) and macroscopic pictures of livers (right panel). D: Liver weight; E: Intrahepatic triglyceride (TG) content. Liver function was evaluated by detecting serum levels of alanine aminotransferase (ALT) (F), aspartate aminotransferase (AST) (G), total cholesterol (H) and uric acid (I). Scale bar in panel represents 100 μm. Data are presented as the mean ± S.D. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* HFD(-) AITC(-), $^{d}P < 0.01$ *vs* HFD(+) AITC(-). HFD: High fat diet; AITC: Allyl isothiocyanate; TG: Triglyceride; H&E: Hematoxylin and eosin; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase.

significantly downregulated *SREBP1* protein levels and its target genes, including *SCD1* and *FAS* (Figure 2A). In contrast, AITC increased the expression levels of proteins involved in fatty acid β-oxidation, such as *PGC1a*, *PPARa* and *CPT1a*, in the livers of HFD-fed mice (Figure 2A).

Previous studies revealed that *Sirt1* activation attenuates hepatic steatosis in mice with diet- or genetics-induced obesity by regulating hepatic lipogenesis and fatty acid oxidation[16,29,30]. To investigate whether Sirt1/AMPK are involved in the amelioration of the HFD-induced dysregulation of hepatic lipid homeostasis by AITC, we evaluated *Sirt1* and p-*AMPKa* expression levels *in vivo*. As shown in Figure 2B, AITC treatment markedly elevated hepatic protein levels of Sirt1 and p-AMPKα. Collectively, these results suggest that AITC attenuates *de novo* lipogenesis and promotes fatty acid β-oxidation by activating the Sirt1/AMPK signaling pathway *in vivo* in the liver tissues of HFD-fed mice.

AITC decreases HFD-induced inflammation by inhibiting the NF-κB signaling pathway in vivo

In addition to its protective role in lipogenesis and fatty acid β-oxidation, AITC treatment substantially decreased the transcription of proinflammatory cytokines [tumor necrosis factor α (TNFα), interleukin (IL)-1β, and IL-6] in the liver tissues of HFD-fed mice (Figure 3A).

To investigate whether IKK/NF-κB are involved in the amelioration of hepatic inflammation by AITC, the expression levels of IKK/NF-κB signaling pathway components were detected. As shown in Figure 3B, AITC treatment upregulated *IκBa* protein levels and downregulated IKK, IκBα, and p65 phosphorylation. These results indicate that AITC decreases inflammation by inhibiting the IKK/NF-κB signaling pathway *in vivo*.

AITC alleviates PA-induced lipid accumulation in hepatocytes

Our above data revealed that AITC could ameliorate hepatic steatosis *in vivo*. To investigate the effect of AITC on PA-induced lipid deposition *in vitro*, we used AML-12 cells, which have been well documented as cellular models of NAFLD[31,32]. First, we evaluated the viability of AML-12 cells after AITC treatment. The cultured hepatocytes were treated with different concentrations of AITC for 24 h, and cell viability was measured using cholecystokinin-8 and lactate dehydrogenase release assays. Finally, we chose 20 μM as the optimal AITC concentration for subsequent experiments because this concentration did not significantly affect cell viability (Figure 4A and B). Then, PA-stimulated AML-12 cells were treated with AITC (20 μmol/L) or vehicle for 24 h. As shown in Figure 4C and D, AITC relieved the PA-induced increases in intracellular TG levels and lipid accumulation in AML-12 cells. These data demonstrate that AITC directly alleviates PA-induced lipid accumulation in hepatocytes.

AITC attenuates de novo lipogenesis and promotes fatty acid β-oxidation by activating the Sirt1/AMPK signaling pathway in vitro

Consistent with the *in vivo* findings, AITC treatment significantly decreased the protein levels of SREBP1 and its target genes, including *SCD1*, *FAS* and acetyl-CoA carboxylase (*ACC*), in PA-treated AML-12 cells (Figure 5A). In addition, AITC enhanced *PGC1a* expression in PA-treated AML-12 cells (Figure 5C).

As shown in Figure 5B *in vitro*, AITC upregulated *Sirt1* and p-*AMPKa* levels, consistent with the *in vivo* results (Figure 6A). These results indicate that AITC attenuates lipogenesis and promotes fatty acid β-oxidation by activating the Sirt1/AMPK signaling pathway *in vitro*.

AITC attenuates inflammation by inhibiting the NF-κB signaling pathway in vitro

Consistent with the *in vivo* findings, AITC treatment significantly decreased *TNFa* and *IL-6* mRNA levels in PA-treated AML-12 cells (Figure 6A). As shown in Figure 6B, AITC upregulated IκBα protein levels and downregulated IKK, IκBα, and p65 phosphorylation in PA-stimulated AML-12 cells. Taken together, these findings clearly indicate that AITC may attenuate inflammation by inhibiting the *NF-κB* signaling pathway *in vitro*.

Sirt1 or AMPKα knockdown abolishes the ability of AITC to mitigate TG levels

To investigate whether AITC can alleviate lipid accumulation through the Sirt1/AMPK pathway, Sirt1 expression in AML-12 cells was selectively knocked down by siRNA transfection (Figure 7A). Sirt1 knockdown abolished the ability of AITC to ameliorate TG accumulation and p-AMPKα and PGC1α upregulation induced by PA in AML-12 cells (Figure 7B and C). Consistently, AMPKα knockdown significantly reversed the effects of AITC on PA-induced intracellular TG

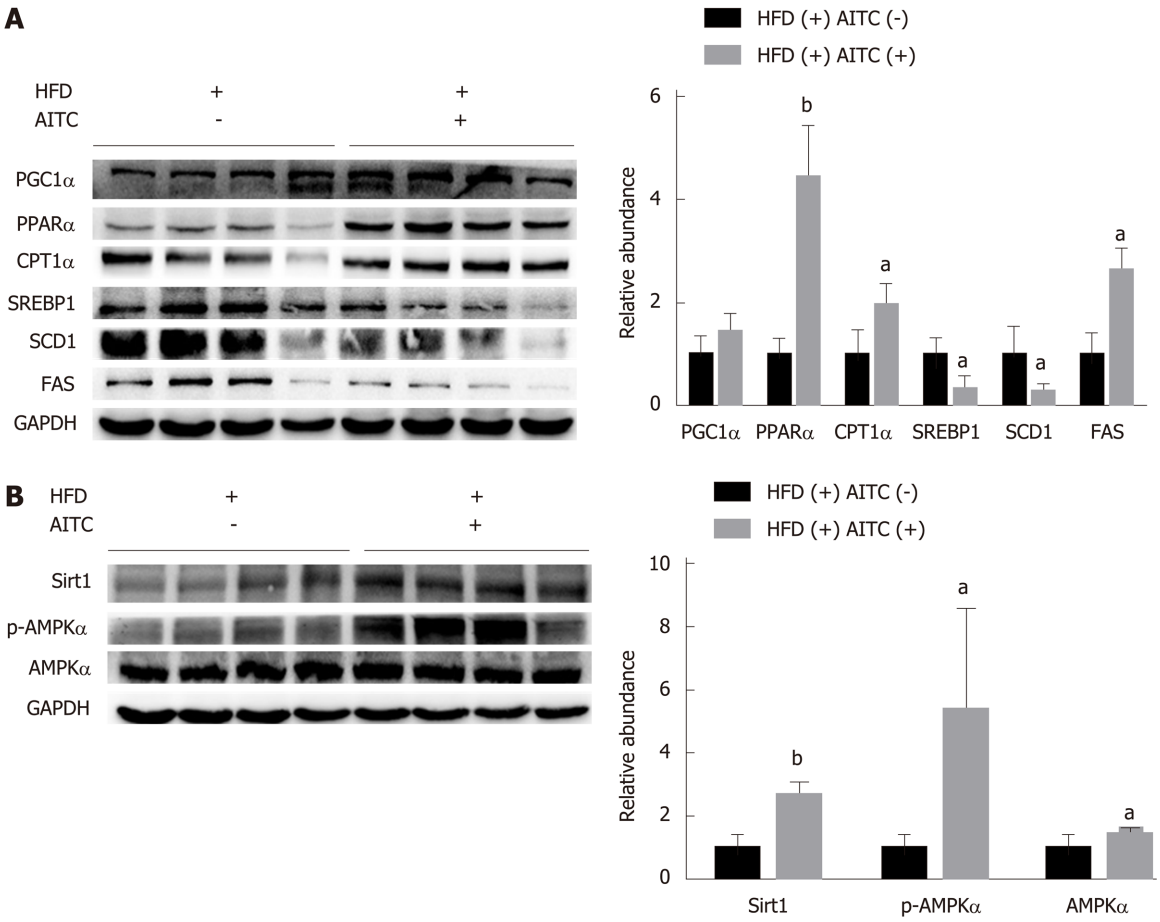

Figure 2 Allyl isothiocyanate upregulates the expression of proteins involved in fatty acid β-oxidation, downregulates the protein levels of lipogenesis genes, and activates the Sirtuin 1/AMP-activated protein kinase signaling pathway in the liver tissues of high fat diet-fed mice. A: The protein expression of sterol regulatory elementbinding protein 1 (SREBP1), its lipogenesis target genes (*SCD1* and *FAS*), and genes involved in fatty acid β-oxidation, such as proliferator-activated receptor gamma coactivator 1α (PGC1α), peroxisome proliferator-activated receptor α (PPARα) and carnitine palmitoyltransferase 1 α (CPT1α), was detected by western blot analysis. B: Sirtuin 1 (Sirt1), total and phosphorylated AMP-activated protein kinase α (AMPKα) protein expression was detected by western blot analysis. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* HFD(+) AITC(-). PGC1α: Proliferator-activated receptor gamma coactivator 1α; PPARα: Peroxisome proliferator-activated receptor α; CPT1α: Carnitine palmitoyltransferase 1 α; SREBP1: Sterol regulatory elementbinding protein 1; SCD1: Stearoyl coenzyme A desaturase 1; FAS: Fatty acid synthase; Sirt1: Sirtuin 1; AMPKα: AMP-activated protein kinase α; p-AMPKα: Phosphorylated AMP-activated protein kinase α; HFD: High fat diet; AITC: Allyl isothiocyanate.

accumulation in AML-12 cells (Figure 7D and E). Taken together, these findings verify that AITC ameliorates lipid accumulation by activating the Sirt1/AMPK signaling pathway.

DISCUSSION

In the current study, we investigated the effects and mechanisms of AITC on NAFLD development (summarized in Figure 8A). Our results demonstrate that AITC significantly ameliorates hepatic lipid accumulation by activating the *Sirt1/AMPK* pathway and alleviates hepatic inflammatory responses by inhibiting the *NF-κB* pathway both *in vivo* and *in vitro*.

Some studies suggested that AITC leads to better metabolic outcome. Kim YJ demonstrated that AITC effectively suppresses the expression of genes that are up-regulated during adipogenesis, such as PPARγ, C/EBPα and FAS[27]. Miyata S showed that AITC reduces *de novo* synthesis of both fatty acids and cholesterol in human hepatoma Huh-7 cells[33]. These results indicate a physiological function of AITC in lipid metabolism regulation.

A previous study by Anh *et al*. demonstrated that AITC protects against HFD-induced obesity and insulin resistance in mice and that the protective effect of AITC may be partly mediated through the modulation of mitochondrial dysfunction in skeletal muscle cells and the liver[25]. However, the mechanism underlying the

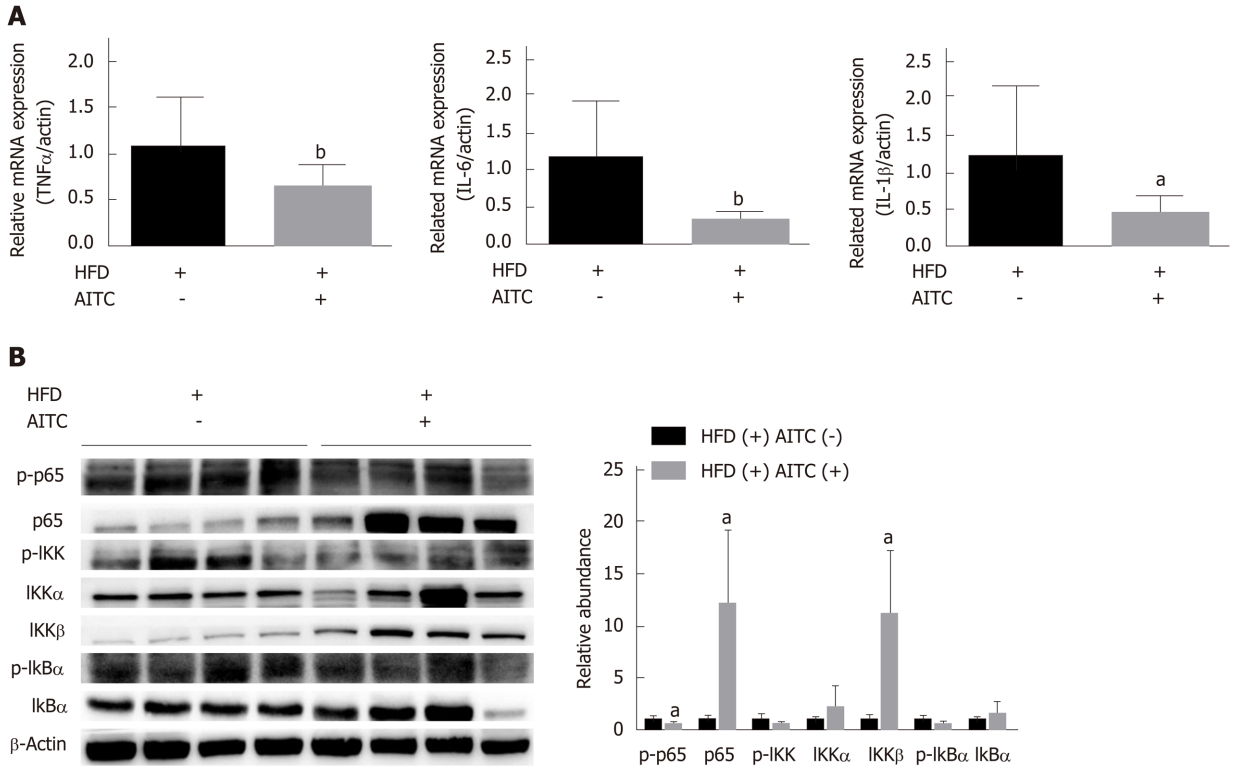

Figure 3 Allyl isothiocyanate attenuates hepatic inflammation and inhibits the I*κB* kinase /nuclear factor kappa B signaling pathway in the liver tissues of high fat diet-fed mice. A: The mRNA levels of proinflammatory cytokines in the liver of high fat diet (HFD)-fed control (n = 9) and HFD-fed allyl isothiocyanate (AITC)-treated mice (n = 10) were measured by quantitative real-time PCR. B: The protein expression of phosphorylated p65, p65, phosphorylated IκB kinase (IKK), IKKα, IKKβ, total and phosphorylated inhibitor of nuclear factor kappa B α (IκB α) in the liver was detected by western blot analysis. Data are presented as the mean ± S.D. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* HFD(+) AITC(-). TNFα: Tumor necrosis factor α; IL-6: Interleukin-6; IL-1β: Interleukin-1β; HFD: High fat diet; AITC: Allyl isothiocyanate; p-p65: Phosphorylated p65; IKK: IκB kinase; IκBα: Inhibitor of nuclear factor kappa B α.

beneficial effects of AITC on hepatic steatosis and liver inflammation has not been clearly defined. Our results suggested that AITC ameliorates lipid accumulation by activating the Sirt1/AMPKα signaling pathway and improves inflammation in hepatocytes both *in vivo* and *in vitro*.

A series of recent studies revealed that Sirt1 plays a critical role in various cellular and physiological processes, including lipid metabolism and energy homeostasis. A previous study demonstrated that Sirt1 transgenic mice are protected from HFD-induced metabolic damage *via* the upregulation of *PGC1a* and the inhibition of the NF-κB pathway[29]. Consistently, another study showed that hepatic overexpression of *Sirt1* attenuates hepatic steatosis, possibly by inhibiting endoplasmic reticulum stress in the liver of obese mice[30]. On the other hand, liver-specific *Sirt1*-knockout mice develop hepatic steatosis, hepatic inflammation, and endoplasmic reticulum stress, which are associated with decreased hepatic fatty acid oxidation and increased lipogenesis[14,15]. In addition, these findings were supported by later reports that pharmacological activation of *Sirt1* protects against HFD-induced metabolic disorders[17,34]. Sirt1 regulates lipid homeostasis through multiple nutrient sensors such as SREBP1, AMPK, PGC1α, PPARα and the hepatocyte-derived hormone fibroblast growth factor 21[14-16,35]. In this study, we found that AITC significantly ameliorated hepatocyte lipid accumulation both *in vivo* and *in vitro* by activating the Sirt1/AMPK signaling pathway, resulting in decreased expression of lipogenesis genes, such as *SREBP1*, *SCD1* and *FAS*, and increased expression of fatty acid β-oxidation genes, including *PGC1a*, *PPARa* and *CPT1a*. Furthermore, another study showed that sulforaphane-induced *Sirt1* activation inhibits endoplasmic reticulum (commonly referred to as ER) stress and prevents cardiomyocytes from hypoxia/reoxygenation injury *in vitro*[36]. As we have detected *Sirt1* activation after AITC application, further studies should be carried out to explore mechanisms of ER stress in this model.

Chronic inflammation is characterized by the abnormal production of proinflammatory cytokines, including TNFα, IL-1β and IL-6, and the activation of inflammatory signaling pathways, such as the NF-κB and JNK pathways; moreover, it has been proposed to play a crucial role in the pathogenesis of NAFLD[7,37]. A number of recent studies have demonstrated a key role for the IKK/NF-κB signaling pathway

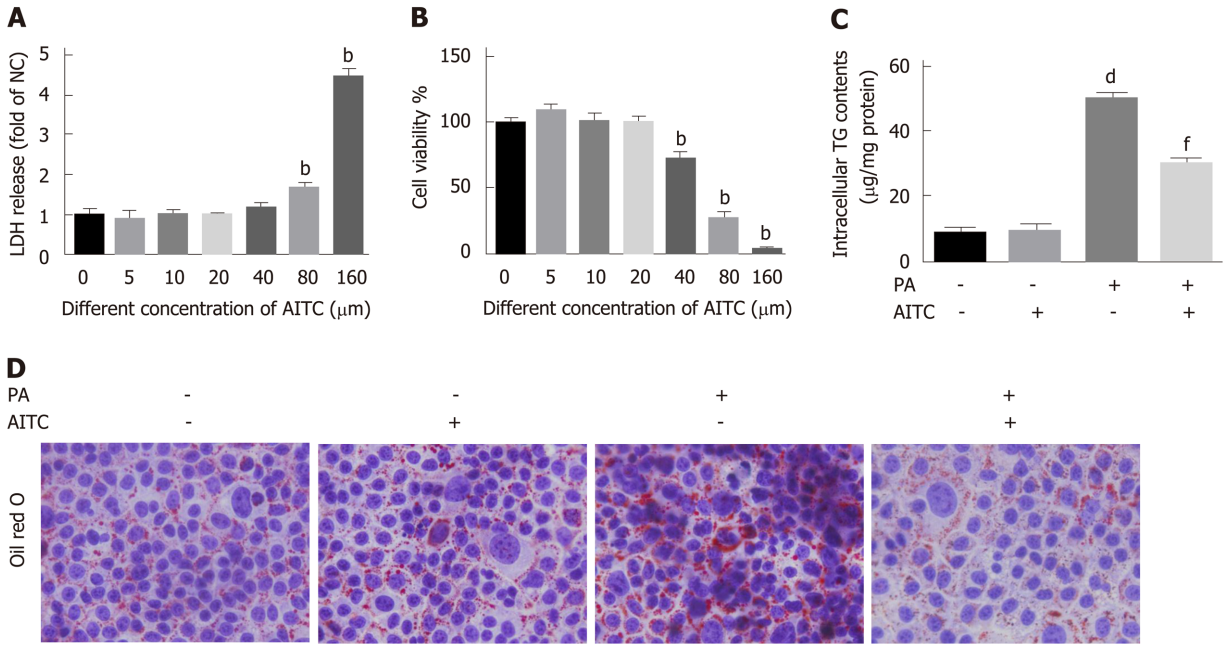

Figure 4 Allyl isothiocyanate alleviates palmitate acid-induced lipid accumulation *in vitro*. Palmitate acid (200 μmo/L)-stimulated AML-12 cells were treated with allyl isothiocyanate (20 μmo/L) or dimethyl sulfoxide (vehicle) for 24 h. A: Cytotoxicity was measured by the lactate dehydrogenase (LDH) release method in AML-12 cells (n = 3/group). B: Cell viability was measured using the cholecystokinin-8 (CCK-8) assay in AML-12 cells (n = 3/group). C: Intracellular triglyceride (TG) content in AML-12 cells (n = 3/group). And D: Representative image of oil red O staining of AML-12 cells in different groups. Scale bar in panel represents 100 μm. Data are presented as the mean ± SD. $^{b}P < 0.01$ *vs* 0 μmol/L AITC, $^{d}P < 0.01$ *vs* PA(-) AITC(-), $^{f}P < 0.01$ *vs* PA(+) AITC(-). LDH: Lactate dehydrogenase; CCK-8: Cholecystokinin-8; TG: Triglyceride; NC: Negative control; PA: Palmitate acid: AITC: Allyl isothiocyanate.

in NAFLD development[8,9]. In response to numerous inflammatory stimuli, *IKK* complex activation induces *IκB* phosphorylation and subsequent degradation, which releases *NF-κB* and allows it to translocate into the nucleus[38]. A previous finding indicated that hepatic lipid accumulation activates the IKK/NF-κB pathway, promoting downstream proinflammatory cytokine production and subacute inflammation[39]. In this study, we demonstrated that AITC treatment upregulated Iκβα protein levels and downregulated IKK, Iκβα, and p65 phosphorylation in both the NAFLD mouse and cellular models. Furthermore, AITC treatment substantially decreased hepatic proinflammatory cytokine levels *in vivo* and *in vitro*. In addition, HFD-induced liver injury was attenuated in AITC-treated mice, as evidenced by markedly decreased serum levels of ALT and AST.

Although several isothiocyanates have been proposed as chemopreventive agents for cancers, AITC has been reported to exhibit both carcinogenic and anticarcinogenic potential. A previous study demonstrated that AITC has the ability to cause Cu(II)-mediated DNA damage and induce 8-oxo-7,8-dihydro-29-deoxyguanosine (8-oxodG) formation, leading to carcinogenesis in human myelogenous leukemic cell lines[40]. Moreover, impaired copper availability in obesity-related NAFLD was shown to predict early atherosclerosis as a main cardiovascular risk[41]. On the other hand, another study demonstrated that AITC could inhibit proliferation of human prostate cancer cells through inducing G2/M arrest and apoptosis[22]. Our studies mentioned above have proposed many different mechanisms of AITC-induced amelioration of hepatic steatosis and inflammation in both i*n vivo* and *in vitro* models of NAFLD, but none have explored whether AITC took part in Cu(II)-mediated DNA damage or cancer development. Further studies are required to clarify whether AITC induces oxidative DNA damage in our model and investigate the mechanism of AITC on cancer.

In conclusion, our study demonstrates that AITC ameliorates hepatic steatosis and inflammation by activating the Sirt1/AMPK and inhibiting the IKK/NF-κB signaling pathway, respectively. This study indicates that AITC may be a potential therapeutic agent for the progress of NAFLD.

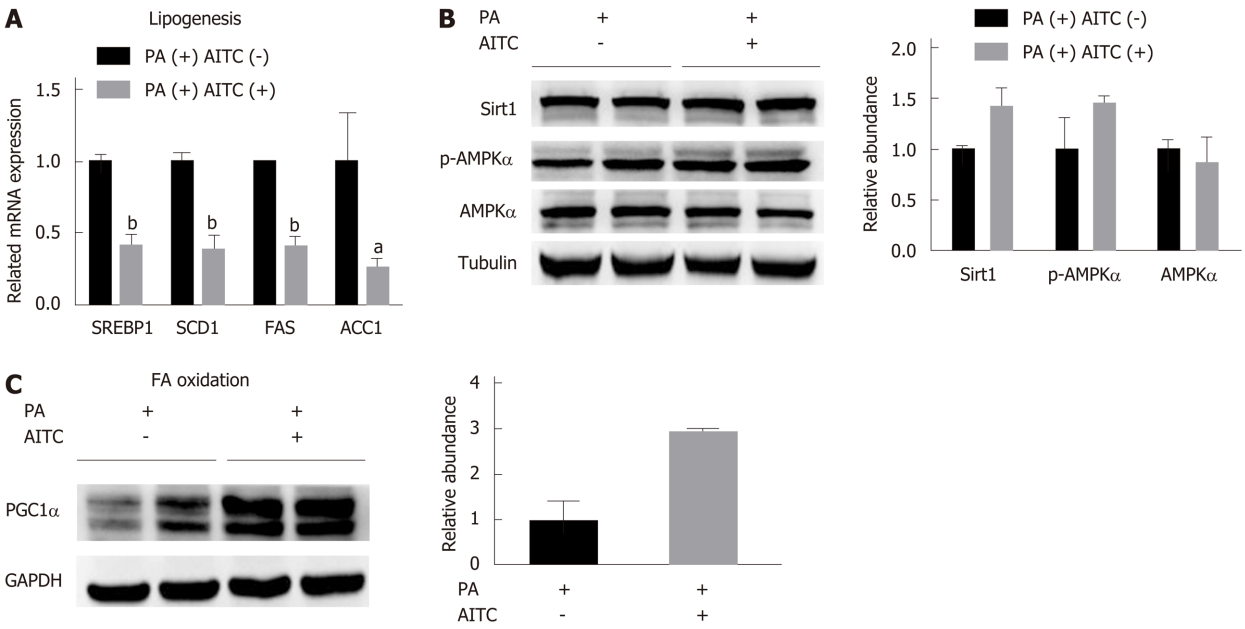

Figure 5 Allyl isothiocyanate downregulates the mRNA levels of genes involved in lipogenesis, upregulates the mRNA levels of genes involved in fatty acid β-oxidation and activates the Sirtuin 1/AMP-activated protein kinase signaling pathway *in vitro*. A: Palmitate acid (PA) (200 μmol/L)-stimulated AML-12 cells were treated with allyl isothiocyanate (AITC) (20 μmol/L) or dimethyl sulfoxide (DMSO) (vehicle) for 6 h (n = 3/group). The mRNA levels of sterol regulatory elementbinding protein 1 (SREBP1) and its lipogenesis target genes, including stearoyl coenzyme A desaturase 1 (SCD1), fatty acid synthase (FAS) and acetyl-CoA carboxylase 1 (ACC1), were determined. B: PA (200 μmol/L)-stimulated AML-12 cells were treated with AITC (20 μmol/L) or DMSO (vehicle) for 24 h. The protein expression of Sirtuin 1 (Sirt1), total and phosphorylated AMP-activated protein kinase α (AMPKα) was detected by western blot analysis. C: PA (200 μmol/L)-stimulated AML-12 cells were treated with AITC (20 μmol/L) or DMSO (vehicle) for 24 h. The protein level of proliferator-activated receptor gamma coactivator1α (PGC1α), which is involved in fatty acid β-oxidation, was detected by western blot analysis. Data are presented as the mean ± SD. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* PA(+) AITC(-). PA: Palmitate acid; AITC: Allyl isothiocyanate; PGC1α: Proliferator-activated receptor gamma coactivator 1α; SREBP1: Sterol regulatory elementbinding protein 1; SCD1: Stearoyl coenzyme A desaturase 1; FAS: Fatty acid synthase; ACC1: Acetyl-CoA carboxylase 1; Sirt1: Sirtuin 1; AMPKα: AMP-activated protein kinase α; p-AMPKα: Phosphorylated AMP-activated protein kinase α.

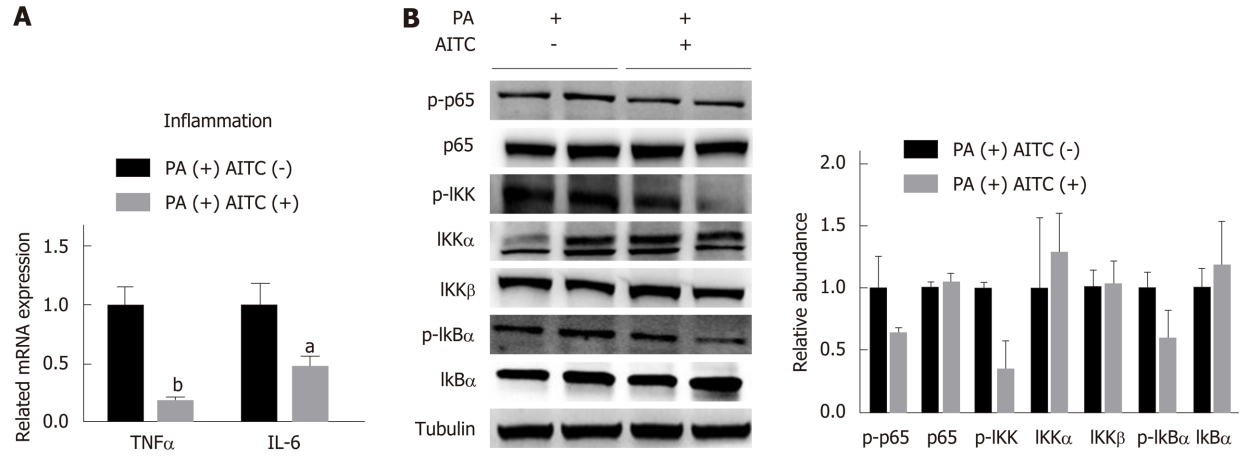

Figure 6 Allyl isothiocyanate downregulates the mRNA levels of proinflammatory markers and inhibits the I*κB* kinase /nuclear factor kappa B signaling pathway *in vitro*. A: Palmitate acid (PA) (200 μmol/L)-stimulated AML-12 cells were treated with allyl isothiocyanate (AITC) (20 μmol/L) or dimethyl sulfoxide (DMSO) (vehicle) for 6 h (n = 3/group). The mRNA levels of proinflammatory cytokines were measured by quantitative real-time PCR. (B) PA (200 μmol/L)-stimulated AML-12 cells were treated with AITC (20 μmol/L) or DMSO (vehicle) for 24 h. The protein expression of phosphorylated p65, p65, phosphorylated IκB kinase (IKK), IKKα, IKKβ, total and phosphorylated inhibitor of nuclear factor kappa B α (IκB α) was detected by western blot analysis. Data are presented as the mean ± S.D. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* PA(+) AITC(-). TNFα: Tumor necrosis factor α; IL-6: Interleukin-6; PA: Palmitate acid; AITC: Allyl isothiocyanate; p-p65: Phosphorylated p65; IKK: IκB kinase; IκBα: Inhibitor of nuclear factor kappa B α.

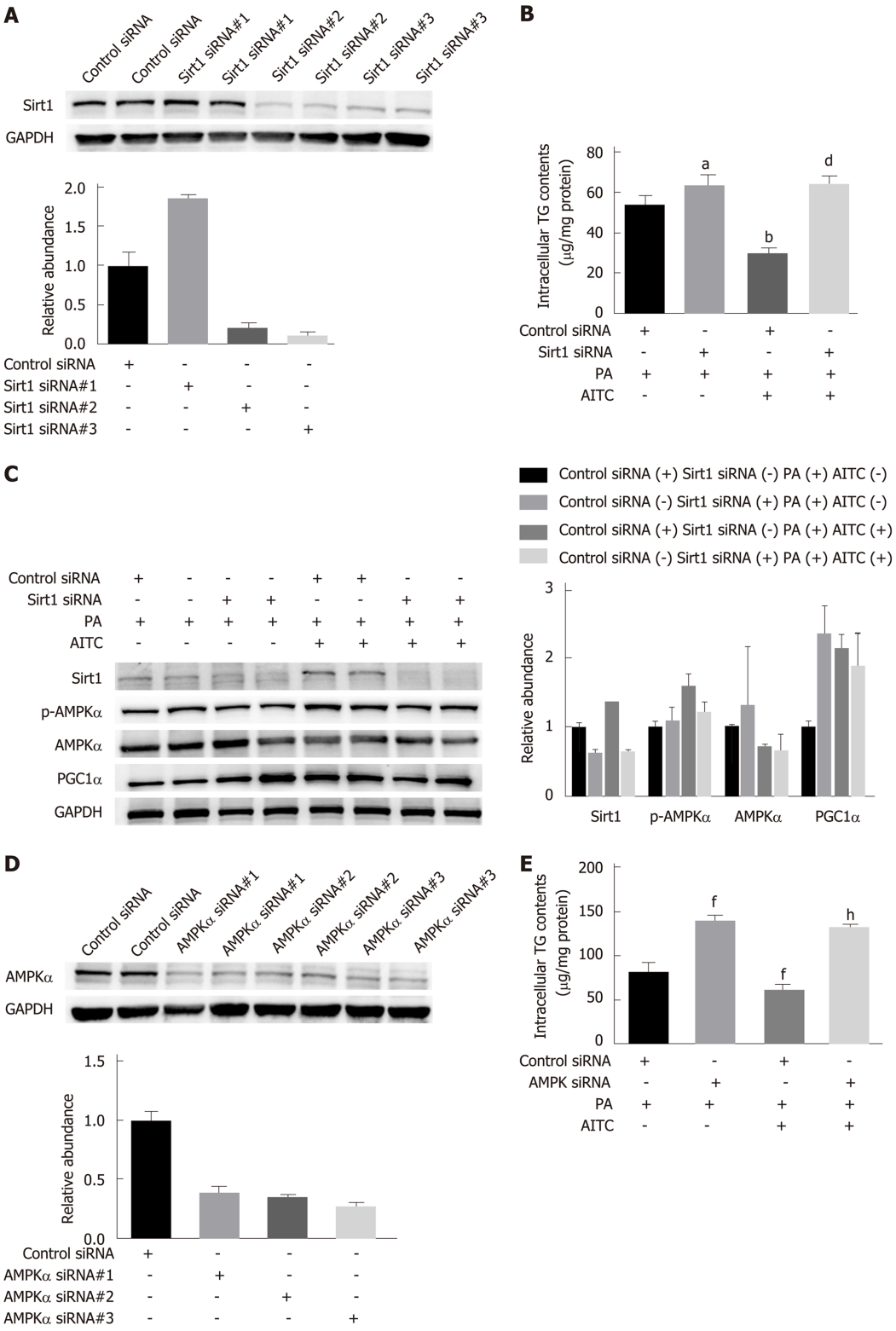

Figure 7 Allyl isothiocyanate ameliorates lipid accumulation by activating the Sirt1/AMPKα signaling pathway. After transfection with Sirtuin 1 (Sirt1) small interfering RNA (siRNA), AMP-activated protein kinase α (AMPKα) siRNA or the corresponding scrambled control for 48 h, AML-12 cells were incubated in normal medium or medium containing palmitate acid (PA) with or without allyl isothiocyanate (AITC) for 24 h. A: Three different Sirt1 siRNA sequences were used, and Sirt1 protein levels were examined by western blot analysis. In the following experiments, we selected Sirt1 siRNA #3. B: Intracellular triglyceride (TG) content in AML-12 cells (n = 4/group). C: Sirt1, phosphorylated AMPKα and proliferator-activated receptor gamma coactivator1α (PGC1α) protein expression was detected by western blot analysis. D: Three different AMPKα siRNA sequences were used, and AMPKα protein levels were examined by western blot analysis. In the subsequent experiments, we selected AMPKα siRNA #3. (E) Intracellular TG content in AML-12 cells (n = 4/group). Data are presented as the mean ± SD. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* Control siRNA + PA, $^{d}P < 0.01$ *vs* Control siRNA + PA + AITC, $^{f}P < 0.01$ *vs* Control siRNA + PA, $^{h}P < 0.01$ *vs* Control siRNA + PA + AITC. PA: Palmitate acid; AITC: Allyl isothiocyanate; PGC1α: Proliferator-activated receptor gamma coactivator 1α; Sirt1: Sirtuin 1; AMPKα: AMP-activated protein kinase α; p-AMPKα: Phosphorylated AMP-activated protein kinase α; TG: Triglyceride.

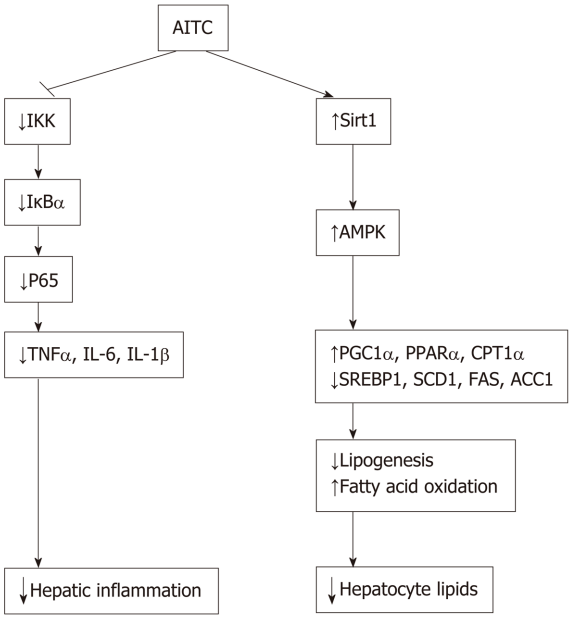

Figure 8 Model of allyl isothiocyanate action. Schematic diagram: allyl isothiocyanate ameliorates hepatic lipid accumulation and hepatic inflammation by activating the Sirt1/AMPK signaling pathway and inhibiting the NF-κB pathway. AITC: Allyl isothiocyanate; IKK: IκB kinase; IκBα: Inhibitor of nuclear factor kappa B α; TNFα: Tumor necrosis factor α; IL-6: Interleukin-6; IL-1β: Interleukin-1β; Sirt1: Sirtuin 1; AMPKα: AMP-activated protein kinase α; PGC1α: Proliferator-activated receptor gamma coactivator 1α; PPARα: Peroxisome proliferator-activated receptor α; CPT1α: Carnitine palmitoyltransferase 1 α; SREBP1: Sterol regulatory elementbinding protein 1; SCD1: Stearoyl coenzyme A desaturase 1; FAS: Fatty acid synthase; ACC1: Acetyl-CoA carboxylase 1.

ARTICLE HIGHLIGHTS

Research background

Nonalcoholic fatty liver disease (NAFLD) is an unmet medical need with no approved therapies. Recent studies have shown that allyl isothiocyanate (AITC) has a potential protective effect on obesity and insulin resistance. The evaluation of the effect of AITC on NAFLD as well as the mechanism of action may provide a new therapeutic trend.

Research motivation

Emerging evidence suggests a beneficial role for AITC in inflammation, cancer, diet-induced obesity and insulin resistance. Enhanced lipolysis in adipocytes and intensified hydrolysis of triglyceride in the serum of rats treated with AITC was also reported. As little is known about its direct impact on liver or its underlying mechanism, it is imperative to characterize the potential effect of AITC on NAFLD.

Research objectives

To validate the effect of AITC on NAFLD and clarify the possible mechanism of action.

Research methods

C57BL/6 mice were fed a high fat diet (HFD) for 8 wk, and AML-12 cells were treated with 200 μmol/L palmitate acid (PA) for 24 h to establish *in vivo* and *in vitro* models of hepatic steatosis. Mice were administered AITC (100 mg/kg/d) orally and AML-12 cells were treated with AITC (20 μmol/L) to detect the effect of AITC on NAFLD.

Research results

Our results show that AITC significantly ameliorates HFD-induced weight gain, hepatic lipid accumulation, inflammation, and PA-induced lipid accumulation as well as inflammation in AML-12 cells, accompanied by activated Sirt1/AMPK and inhibited NF-κB signaling pathways. The curative effect of AITC on lipid accumulation is abolished by siRNA-mediated knockdown of either Sirt1 or AMPKα in AML-12 cells.

Research conclusions

AITC treatment protects against HFD and PA-induced lipid accumulation and inflammation *in vivo* and *in vitro*. These effects are associated with Sirt1/AMPK and NF-κB signaling pathways.

Research perspectives

Plant compounds such as AITC should be further explored for their potential effective activity in NAFLD.

REFERENCES

1 **Younossi ZM**, Koenig AB, Abdelatif D, Fazel Y, Henry L, Wymer M. Global epidemiology of nonalcoholic fatty liver disease-Meta-analytic assessment of prevalence, incidence, and outcomes. *Hepatology* 2016; **64**: 73-84 [PMID: 26707365 DOI: 10.1002/hep.28431]

2 **Cohen JC**, Horton JD, Hobbs HH. Human fatty liver disease: old questions and new insights. *Science* 2011; **332**: 1519-1523 [PMID: 21700865 DOI: 10.1126/science.1204265]

3 **Chalasani N**, Younossi Z, Lavine JE, Diehl AM, Brunt EM, Cusi K, Charlton M, Sanyal AJ. The diagnosis and management of non-alcoholic fatty liver disease: practice Guideline by the American Association for the Study of Liver Diseases, American College of Gastroenterology, and the American Gastroenterological Association. *Hepatology* 2012; **55**: 2005-2023 [PMID: 22488764 DOI: 10.1002/hep.25762]

4 **Sinn DH**, Cho SJ, Gu S, Seong D, Kang D, Kim H, Yi BK, Paik SW, Guallar E, Cho J, Gwak GY. Persistent Nonalcoholic Fatty Liver Disease Increases Risk for Carotid Atherosclerosis. *Gastroenterology* 2016; **151**: 481-488.e1 [PMID: 27283259 DOI: 10.1053/j.gastro.2016.06.001]

5 **Musso G**, Gambino R, Tabibian JH, Ekstedt M, Kechagias S, Hamaguchi M, Hultcrantz R, Hagström H, Yoon SK, Charatcharoenwitthaya P, George J, Barrera F, Hafliðadóttir S, Björnsson ES, Armstrong MJ, Hopkins LJ, Gao X, Francque S, Verrijken A, Yilmaz Y, Lindor KD, Charlton M, Haring R, Lerch MM, Rettig R, Völzke H, Ryu S, Li G, Wong LL, Machado M, Cortez-Pinto H, Yasui K, Cassader M. Association of non-alcoholic fatty liver disease with chronic kidney disease: a systematic review and meta-analysis. *PLoS Med* 2014; **11**: e1001680 [PMID: 25050550 DOI: 10.1371/journal.pmed.1001680]

6 **Younossi ZM**, Loomba R, Rinella ME, Bugianesi E, Marchesini G, Neuschwander-Tetri BA, Serfaty L, Negro F, Caldwell SH, Ratziu V, Corey KE, Friedman SL, Abdelmalek MF, Harrison SA, Sanyal AJ, Lavine JE, Mathurin P, Charlton MR, Chalasani NP, Anstee QM, Kowdley KV, George J, Goodman ZD, Lindor K. Current and future therapeutic regimens for nonalcoholic fatty liver disease and nonalcoholic steatohepatitis. *Hepatology* 2018; **68**: 361-371 [PMID: 29222911 DOI: 10.1002/hep.29724]

7 **Samuel VT**, Shulman GI. Mechanisms for insulin resistance: common threads and missing links. *Cell* 2012; **148**: 852-871 [PMID: 22385956 DOI: 10.1016/j.cell.2012.02.017]

8 **Wang XA**, Zhang R, She ZG, Zhang XF, Jiang DS, Wang T, Gao L, Deng W, Zhang SM, Zhu LH, Guo S, Chen K, Zhang XD, Liu DP, Li H. Interferon regulatory factor 3 constrains IKKβ/NF-κB signaling to alleviate hepatic steatosis and insulin resistance. *Hepatology* 2014; **59**: 870-885 [PMID: 24123166 DOI: 10.1002/hep.26751]

9 **Arkan MC**, Hevener AL, Greten FR, Maeda S, Li ZW, Long JM, Wynshaw-Boris A, Poli G, Olefsky J, Karin M. IKK-beta links inflammation to obesity-induced insulin resistance. *Nat Med* 2005; **11**: 191-198 [PMID: 15685170 DOI: 10.1038/nm1185]

10 **Wang PX**, Zhang XJ, Luo P, Jiang X, Zhang P, Guo J, Zhao GN, Zhu X, Zhang Y, Yang S, Li H. Hepatocyte TRAF3 promotes liver steatosis and systemic insulin resistance through targeting TAK1-dependent signalling. *Nat Commun* 2016; **7**: 10592 [PMID: 26882989 DOI: 10.1038/ncomms10592]

11 **Picard F**, Kurtev M, Chung N, Topark-Ngarm A, Senawong T, Machado De Oliveira R, Leid M, McBurney MW, Guarente L. Sirt1 promotes fat mobilization in white adipocytes by repressing PPAR-gamma. *Nature* 2004; **429**: 771-776 [PMID: 15175761 DOI: 10.1038/nature02583]

12 **Cohen HY**, Miller C, Bitterman KJ, Wall NR, Hekking B, Kessler B, Howitz KT, Gorospe M, de Cabo R, Sinclair DA. Calorie restriction promotes mammalian cell survival by inducing the SIRT1 deacetylase. *Science* 2004; **305**: 390-392 [PMID: 15205477 DOI: 10.1126/science.1099196]

13 **Herzig S**, Shaw RJ. AMPK: guardian of metabolism and mitochondrial homeostasis. *Nat Rev Mol Cell Biol* 2018; **19**: 121-135 [PMID: 28974774 DOI: 10.1038/nrm.2017.95]

14 **Li Y**, Wong K, Giles A, Jiang J, Lee JW, Adams AC, Kharitonenkov A, Yang Q, Gao B, Guarente L, Zang M. Hepatic SIRT1 attenuates hepatic steatosis and controls energy balance in mice by inducing fibroblast growth factor 21. *Gastroenterology* 2014; **146**: 539-49.e7 [PMID: 24184811 DOI: 10.1053/j.gastro.2013.10.059]

15 **Purushotham A**, Schug TT, Xu Q, Surapureddi S, Guo X, Li X. Hepatocyte-specific deletion of SIRT1 alters fatty acid metabolism and results in hepatic steatosis and inflammation. *Cell Metab* 2009; **9**: 327-338 [PMID: 19356714 DOI: 10.1016/j.cmet.2009.02.006]

16 **Ponugoti B**, Kim DH, Xiao Z, Smith Z, Miao J, Zang M, Wu SY, Chiang CM, Veenstra TD, Kemper JK. SIRT1 deacetylates and inhibits SREBP-1C activity in regulation of hepatic lipid metabolism. *J Biol Chem* 2010; **285**: 33959-33970 [PMID: 20817729 DOI: 10.1074/jbc.M110.122978]

17 **Feige JN**, Lagouge M, Canto C, Strehle A, Houten SM, Milne JC, Lambert PD, Mataki C, Elliott PJ, Auwerx J. Specific SIRT1 activation mimics low energy levels and protects against diet-induced metabolic disorders by enhancing fat oxidation. *Cell Metab* 2008; **8**: 347-358 [PMID: 19046567 DOI: 10.1016/j.cmet.2008.08.017]

18 **Dogra S**, Kar AK, Girdhar K, Daniel PV, Chatterjee S, Choubey A, Ghosh S, Patnaik S, Ghosh D, Mondal P. Zinc oxide nanoparticles attenuate hepatic steatosis development in high-fat-diet fed mice through activated AMPK signaling axis. *Nanomedicine* 2019; **17**: 210-222 [PMID: 30708053 DOI: 10.1016/j.nano.2019.01.013]

19 **Liou CJ**, Wei CH, Chen YL, Cheng CY, Wang CL, Huang WC. Fisetin Protects Against Hepatic Steatosis Through Regulation of the Sirt1/AMPK and Fatty Acid β-Oxidation Signaling Pathway in High-Fat Diet-Induced Obese Mice. *Cell Physiol Biochem* 2018; **49**: 1870-1884 [PMID: 30235452 DOI: 10.1159/000493650]

20 **Zhang Y**. Allyl isothiocyanate as a cancer chemopreventive phytochemical. *Mol Nutr Food Res* 2010; **54**: 127-135 [PMID: 19960458 DOI: 10.1002/mnfr.200900323]

21 **Getahun SM**, Chung FL. Conversion of glucosinolates to isothiocyanates in humans after ingestion of cooked watercress. *Cancer Epidemiol Biomarkers Prev* 1999; **8**: 447-451 [PMID: 10350441]

22 **Xiao D**, Srivastava SK, Lew KL, Zeng Y, Hershberger P, Johnson CS, Trump DL, Singh SV. Allyl isothiocyanate, a constituent of cruciferous vegetables, inhibits proliferation of human prostate cancer cells by causing G2/M arrest and inducing apoptosis. *Carcinogenesis* 2003; **24**: 891-897 [PMID: 12771033 DOI: 10.1093/carcin/bgg023]

23 **Jeong WS**, Kim IW, Hu R, Kong AN. Modulatory properties of various natural chemopreventive agents on the activation of NF-kappaB signaling pathway. *Pharm Res* 2004; **21**: 661-670 [PMID: 15139523]

24 **Wagner AE**, Boesch-Saadatmandi C, Dose J, Schultheiss G, Rimbach G. Anti-inflammatory potential of allyl-isothiocyanate--role of Nrf2, NF-(κ) B and microRNA-155. *J Cell Mol Med* 2012; **16**: 836-843 [PMID: 21692985 DOI: 10.1111/j.1582-4934.2011.01367.x]

25 **Ahn J**, Lee H, Im SW, Jung CH, Ha TY. Allyl isothiocyanate ameliorates insulin resistance through the regulation of mitochondrial function. *J Nutr Biochem* 2014; **25**: 1026-1034 [PMID: 25034503 DOI: 10.1016/j.jnutbio.2014.05.006]

26 **Okulicz M**. Multidirectional time-dependent effect of sinigrin and allyl isothiocyanate on metabolic parameters in rats. *Plant Foods Hum Nutr* 2010; **65**: 217-224 [PMID: 20809411 DOI: 10.1007/s11130-010-0183-3]

27 **Kim YJ**, Lee DH, Ahn J, Chung WJ, Jang YJ, Seong KS, Moon JH, Ha TY, Jung CH. Pharmacokinetics, Tissue Distribution, and Anti-Lipogenic/Adipogenic Effects of Allyl-Isothiocyanate Metabolites. *PLoS One* 2015; **10**: e0132151 [PMID: 26317351 DOI: 10.1371/journal.pone.0132151]

28 **Shimano H**, Sato R. SREBP-regulated lipid metabolism: convergent physiology - divergent pathophysiology. *Nat Rev Endocrinol* 2017; **13**: 710-730 [PMID: 28849786 DOI: 10.1038/nrendo.2017.91]

29 **Pfluger PT**, Herranz D, Velasco-Miguel S, Serrano M, Tschöp MH. Sirt1 protects against high-fat diet-induced metabolic damage. *Proc Natl Acad Sci USA* 2008; **105**: 9793-9798 [PMID: 18599449 DOI: 10.1073/pnas.0802917105]

30 **Li Y**, Xu S, Giles A, Nakamura K, Lee JW, Hou X, Donmez G, Li J, Luo Z, Walsh K, Guarente L, Zang M. Hepatic overexpression of SIRT1 in mice attenuates endoplasmic reticulum stress and insulin resistance in the liver. *FASEB J* 2011; **25**: 1664-1679 [PMID: 21321189 DOI: 10.1096/fj.10-173492]

31 **Yu J**, Chu ES, Wang R, Wang S, Wu CW, Wong VW, Chan HL, Farrell GC, Sung JJ. Heme oxygenase-1 protects against steatohepatitis in both cultured hepatocytes and mice. *Gastroenterology* 2010; **138**: 694-704, 704.e1 [PMID: 19818781 DOI: 10.1053/j.gastro.2009.09.058]

32 **Li S**, Dou X, Ning H, Song Q, Wei W, Zhang X, Shen C, Li J, Sun C, Song Z. Sirtuin 3 acts as a negative regulator of autophagy dictating hepatocyte susceptibility to lipotoxicity. *Hepatology* 2017; **66**: 936-952 [PMID: 28437863 DOI: 10.1002/hep.29229]

33 **Miyata S**, Inoue J, Shimizu M, Sato R. Allyl isothiocyanate suppresses the proteolytic activation of sterol regulatory element-binding proteins and de novo fatty acid and cholesterol synthesis. *Biosci Biotechnol Biochem* 2016; **80**: 1006-1011 [PMID: 26822063 DOI: 10.1080/09168451.2015.1132154]

34 **Lagouge M**, Argmann C, Gerhart-Hines Z, Meziane H, Lerin C, Daussin F, Messadeq N, Milne J, Lambert P, Elliott P, Geny B, Laakso M, Puigserver P, Auwerx J. Resveratrol improves mitochondrial function and protects against metabolic disease by activating SIRT1 and PGC-1alpha. *Cell* 2006; **127**: 1109-1122 [PMID: 17112576 DOI: 10.1016/j.cell.2006.11.013]

35 **Hou X**, Xu S, Maitland-Toolan KA, Sato K, Jiang B, Ido Y, Lan F, Walsh K, Wierzbicki M, Verbeuren TJ, Cohen RA, Zang M. SIRT1 regulates hepatocyte lipid metabolism through activating AMP-activated protein kinase. *J Biol Chem* 2008; **283**: 20015-20026 [PMID: 18482975 DOI: 10.1074/jbc.M802187200]

36 **Li YP**, Wang SL, Liu B, Tang L, Kuang RR, Wang XB, Zhao C, Song XD, Cao XM, Wu X, Yang PZ, Wang LZ, Chen AH. Sulforaphane prevents rat cardiomyocytes from hypoxia/reoxygenation injury in vitro via activating SIRT1 and subsequently inhibiting ER stress. *Acta Pharmacol Sin* 2016; **37**: 344-353 [PMID: 26775664 DOI: 10.1038/aps.2015.130]

37 **Hotamisligil GS**. Inflammation and metabolic disorders. *Nature* 2006; **444**: 860-867 [PMID: 17167474 DOI: 10.1038/nature05485]

38 **Perkins ND**. Integrating cell-signalling pathways with NF-kappaB and IKK function. *Nat Rev Mol Cell Biol* 2007; **8**: 49-62 [PMID: 17183360 DOI: 10.1038/nrm2083]

39 **Cai D**, Yuan M, Frantz DF, Melendez PA, Hansen L, Lee J, Shoelson SE. Local and systemic insulin resistance resulting from hepatic activation of IKK-beta and NF-kappaB. *Nat Med* 2005; **11**: 183-190 [PMID: 15685173 DOI: 10.1038/nm1166]

40 **Murata M**, Yamashita N, Inoue S, Kawanishi S. Mechanism of oxidative DNA damage induced by carcinogenic allyl isothiocyanate. *Free Radic Biol Med* 2000; **28**: 797-805 [PMID: 10754276]

41 **Tarantino G**, Porcu C, Arciello M, Andreozzi P, Balsano C. Prediction of carotid intima-media thickness in obese patients with low prevalence of comorbidities by serum copper bioavailability. *J Gastroenterol Hepatol* 2018; **33**: 1511-1517 [PMID: 29405466 DOI: 10.1111/jgh.14104]

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ORIGINAL ARTICLE

Basic Study

Effect of Tong Xie Yao Fang on endogenous metabolites in urine of irritable bowel syndrome model rats

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Author contributions: Zhao XY and Wang JW performed most of the experiments, analyzed the experimental results, interpreted them and wrote the manuscript; Yin Y, Li K, Zhang M and Yan FP performed some experiments and interpretation of the results; Wang JW participated in the testing factor design and revised the manuscript. She is also the corresponding author; All co-authors participated in writing and checking the manuscript and approved the submitted manuscript.

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Abstract

BACKGROUND

Tong Xie Yao Fang is a representative traditional Chinese prescription for the treatment of liver and spleen deficiency, abdominal pain and diarrhea. It has a unique function in the treatment of gastrointestinal dysfunction including irritable bowel syndrome (IBS), is a common functional bowel disease. Its main symptoms are recurrent abdominal pain, diarrhea, constipation or alternations between diarrhea and constipation.

There are obvious differences in metabolites between TCM syndromes. By comparing the body fluid metabolism maps of model animals, metabolomics can discover disease biomarkers, analyze the differences in metabolic pathways and understand the pathological process and the metabolic pathways of substances in the body. Thus, the evaluation of animal models tends to be comprehensive and objective. This may provide further understanding between the interaction between Tong Xie Yao Fang and the IBS model.

AIM

To evaluate the effect of Tong Xie Yao Fang on IBS rats by using metabolomics method.

METHODS

Wistar rats were used to establish IBS models, and then randomly divided into four groups: A model control group and three Tong Xie Yao Fang treatment groups (high, medium and low doses). A normal, non-IBS group was established. The rats were treated for 2 wk. On days 0 and 14 of the experimental model, urine was collected for 12 h and was analyzed by ultra-performance liquid chromatography quadrupole time-of-flight mass spectrometry. Nine potential biomarkers were identified, and six major metabolic pathways were found to be related to IBS.

RESULTS

Technology Project of Traditional Chinese Medicine Department of Heilongjiang Province, No. ZQG-034.

Institutional review board statement: This study was approved by the Ethics Committee of the Heilongjiang University of Chinese Medicine.

Institutional animal care and use committee statement: This study was approved by the animal care and use committee of the Heilongjiang University of Chinese Medicine.

Conflict-of-interest statement: Authors declare no conflicts of interest.

Data sharing statement: The detailed experimental methods are available from the corresponding author at xueyingzhao2010@126.com and wangjianwei140918@126.com.

ARRIVE guidelines statement: The ARRIVE Guidelines have been adopted.

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In the study of metabonomics, nine potential biomarkers including L-serine, 4-methylgallic acid, L-threonine, succinylacetone, prolyl-hydroxyproline, valyl-serine, acetyl citrate, marmesin rutinoside and 5-hydroxy-L-tryptophan were identified in urine, which were assigned to amino acids, organic acids, succinyl and glycosides. Furthermore, the metabolic pathway of L-serine, L-threonine and 5-hydroxy-L-tryptophan was found in the Kyoto Encyclopedia of Genes and Genomes, which mainly involved the metabolism of cysteine and methionine, vitamin B6 metabolism, serotonin synapse, tryptophan metabolism, sphingolipid metabolism, digestion, absorption of protein and amino acid metabolism. These pathways are related to intestinal dysfunction, inflammatory syndrome, nervous system dysfunction and other diseases.

CONCLUSION

Tong Xie Yao Fang has pharmacological effects on IBS, and its mechanism may be related to the metabolism of the nine potential biomarkers identified above in urine.

Key words: Tong Xie Yao Fang; Irritable bowel syndrome; Liver-spleen disharmony; Endogenous metabolites; Metabolomics

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Core tip: Effects of Tong Xie Yao Fang on endogenous metabolites in urine of irritable bowel syndrome model rats were investigated through ultra-performance liquid chromatography quadrupole time-of-flight mass spectrometry. Tong Xie Yao Fang has the function of callback endogenous metabolite. Nine potential biomarkers were identified, and six main metabolic pathways were analyzed. They were related to neurotransmitter metabolism, inflammatory immunity, emotional changes and energy metabolism in irritable bowel syndrome disease, which may be the biological basis of irritable bowel syndrome spleen deficiency and liver hyperactivity syndrome.

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INTRODUCTION

Tong Xie Yao Fang is a representative traditional Chinese prescription for the treatment of liver and spleen deficiency, abdominal pain and diarrhea. This prescription is composed of *Atractylodis Macrocephalae Rhizoma* (fried), *Paeoniae Radix Alba* (fried), *Citri Reticulatae Pericarpium* (fried) and Saposhnikoviae Radix (fried). It has a unique function in the treatment of gastrointestinal dysfunction including irritable bowel syndrome (IBS)[1,2].

IBS is a common functional bowel disease, and its main symptoms are recurrent abdominal pain, diarrhea, constipation or alternations between diarrhea and constipation. It is believed that the pathogenesis of IBS involves abnormal interaction between the brain and intestine and is a type of biological-psychological-social disorder. The pathogenesis of IBS is complex and varied, and its pathophysiological basis is mainly gastrointestinal motility and visceral sensory abnormalities.

Animal models of IBS are mostly duplicated from the perspective of emotion, chemical stimulation, physical stimulation and other causes. However, there is currently no unified and recognized method for animal models combining IBS with spleen deficiency and liver hyperactivity. Research in traditional Chinese medicine (TCM) and its mechanism of action related to IBS lacks systematisms and consistency, and further research is restricted. Whether the animal models reflect the characteristics of IBS determines the reasonableness of the research results. Therefore, how to verify IBS in animal models has become an important factor in research. This is based on the function and responsiveness of prescriptions for various syndromes to evaluate the correlation between prescriptions and syndromes[3,4]. Prescription

medicine is the carrier of syndrome differentiation and disease syndrome. We can verify syndrome with a prescription to estimate the correlation between the syndrome and the prescription[5].

However, it is worth thinking about how to expound the overall effect of prescription drugs on disease and syndromes in a comprehensive and systematic way. The complexity of a syndrome has brought many difficulties to the combination of disease and syndrome and correlations between prescription and syndrome. By studying the whole change in small molecule metabolites, metabolomics can restore the related biological events, thus revealing the essence of an organism's biological state and the mechanism of drug action. Metabolomics has the characteristics of systemic methodology such as overall dynamics, synthesis and analysis and has similar characteristics to the overall concept of TCM treatment of diseases[6,7].

There are obvious differences in metabolites between TCM syndromes[8]. By comparing the body fluid metabolism maps of model animals, metabolomics can discover disease biomarkers, analyze the differences in metabolic pathways and understand the pathological process and the metabolic pathways of substances in the body. Thus, the evaluation of animal models tends to be comprehensive and objective[9]. Prescription drugs can play a special role in some of the metabolic pathways in which these markers are located causing the metabolic network to return to normal or improve. Through the combination of metabolomics and correlation between the syndrome and the prescription theory, the mechanism and essence of the animal model are clearly defined.

Therefore, by combining systematic biology such as metabonomics with the correlation of TCM prescription and syndromes, the attributes of animal models can be clearly defined. Through the effect of the prescription on the model, the prescription is used to determine the model of the syndrome from the perspective of metabolites in the body verifying the TCM syndrome with a prescription, clarifying the mechanism and essence of the prescription and confirming the correlation between the prescription and syndrome[10]. Thus, the IBS combination model and the representative prescription can be studied as an organic system by metabolomics research methods.

In this study, we evaluated the effect of Tong Xie Yao Fang on an IBS animal model, studied the regulatory effect of the prescription on the model and clarified the difference in the regulation of endogenous metabolites in the IBS model by Tong Xie Yao Fang. Furthermore, their metabolic patterns were established by exploring the biomarkers. Based on the changing rules of substance quality *in vivo*, the function essence of Tong Xie Yao Fang and the syndromes essence of the IBS model were explored.

MATERIALS AND METHODS

Tong Xie Yao Fang components and reagents

Tong Xie Yao Fang was prepared with large head atractylodes rhizome (*Rhizoma Atractylodis Macrocephalae*), white peony root (*Radix Paeoniae Alba*), dried tangerine peel (*Pericarpium Citri Reticulatae*) and divaricate saposhnikovia root (*Radix Saposhnikoviae*), which were purchased from the Second Affiliated Hospital of Heilongjiang University of Chinese Medicine and composed in 6:4:3:2 proportions. Raw components were soaked in a 10 times volume of distilled water for 0.5 h and boiled twice, first for 1.5 h and then for 1 h. Two of the boiled ingredients were filtered, mixed together and concentrated in a 1:1 ratio (100% concentration) and stored at 4 °C for later use. Tong Xie Yao Fang was diluted in distilled water to a concentration of 0.203 (clinically equivalent), 0.406, and 0.812 g/mL and stored at room temperature before use.

The following reagents were used: Acetonitrile (chromatographic grade, A998-4; Thermo Fisher Scientific, Waltham, MA, United States); methanol (chromatographic grade, CAS-67-56-1; Dikma Technologies, Foothill Ranch, CA, United States); formic acid (chromatographic grade, FS0630-015; Tedia, Fairfield, OH, United States); and distilled water (GB19298; Watsons, China).

Instruments

Ultra-performance liquid chromatography quadrupole time-of-flight mass spectrometry (UPLC-Q-TOF-MS) system: Acquity Ultra type UPLC, Triple TOF 5600$^+$ type mass spectrometer equipped with electrospray ionization (AB SCIEX, Redwood City, CA, United States), MassLynxV4.1 workstation (Waters); KDC-40 type low-speed centrifuge (Zhongjia Branch of Keda Chuangxin Co., Ltd., China); D-78532 type centrifuge (Hettich, Tuttligen, Germany); Vortex3000 type whirl mixer (Wiggens,

Germany); Academic UPW SYSTEM (Milli-Q, United States); Forma 995 type ultra-low temperature refrigerator (Thermo Fisher Scientific); B3200S-T type ultrasonic oscillometer (Branson Ultrasonics Corporation, Shanghai, China); KDC-160HR type high speed tabletop refrigerated centrifuge (Zhongjia Branch of Keda Chuangxin, China); SK-1 type swift mixer (Jiangsu Medical Instrument Factory, China); cp2465 type angioplasty balloon (EV3, United States); E206Fr type children catheter (3 mL; Shenyang Bosilin Medical Instrument Co., Ltd., China); and DYM type segmental epidural catheter (1.0 × 1; Yangzhou Huaxia Medical Device Co., Ltd., China).

IBS model replication, grouping and medication

This study was approved by the Ethics Committee of the Heilongjiang University of Chinese Medicine. All animal experimental procedures were performed in accordance with the Regulations for the Administration of Affairs Concerning Experimental Animals approved by the State Council of People's Republic of China. Wistar female and male rats (clean grade), aged 8 d and body weight 11 ± 2 g, were purchased from Heilongjiang University of Chinese Medicine (HDZD P00102006). The mother rats and their newborn rats were fed in the cage together, and they had free access to food and water at room temperature of 21-24 °C with humidity of 50%-60%. By using the rectal dilatation stimulation method[11] combined with the mother and offspring separation method[12] for the newborn rats from age 8 to 12 d, the angioplasty balloon (15 mm long) was inserted into the rectum 2 cm from the end of the balloon to the anus, and the intestinal tract was slowly dilated. The operation was performed twice daily for 1 min each time and at an interval of 30 min. On days 13-17, the rectum was dilated by balloon angioplasty (20 mm long). On days 18-21, the catheter for children was used to expand the rectum, and 0.1 mL water was injected without changing other conditions. After rectal dilatation every day, neonatal rats were placed in cages separated from the maternal rats for 1 h, and then put back into the cages with the maternal rats. After modeling for 2 wk, the abdominal withdrawal reflex model was used to evaluate the success of the IBS model. IBS model rats were randomly divided into four groups by weight with ten rats in each group: A model group (M) and groups treated with high-, medium- and low-dose Tong Xie Yao Fang denoted as G, Z and D groups, respectively. Another ten rats were used as the normal group (K). The rats for G, Z and D groups were given 8.12, 4.06, and 2.03 g/kg Tong Xie Yao Fang decoction, respectively. The rats in the normal group and model group were given with same amount of saline. All groups were treated once daily for 2 wk.

Biological sample collection and processing method

On days 0 and 14 of the experimental model, urine was collected for 12 h, and then centrifuged at 4 °C at 13000 r/min for 10 min. The supernatant was collected as the urine samples and stored in a -80 °C freezer until use. Urine samples were unfrozen at normal temperature. Next, 100 μL urine was centrifuged at 4 °C at 10000 r/min for 10 min, and the supernatant was absorbed into a glass sample bottle.

Metabolomics data collection

Metabolomics data were measured by UPLC-Q-TOF-MS. Chromatographic conditions were as follows: Rapid resolution chromatographic column (2.1 mm × 100 mm, 1.8 μm); flow rate: 0.4 mL/min; column temperature: 30 °C; detector scanning range: 190-400 nm; detection wavelength: 210, 254 and 280 nm; injection volume: 2 μL; mobile phase: 0.1% formic acid aqueous solution (A) and -0.1% formic acid acetonitrile solution (B); gradient elution: 0-3 min, 98%-48% A, 3-10 min, 48%-2% A, 10-12 min, 2%-98% A, 12-14 min and 98% A. Mass spectrometry (MS) conditions were as follows: Positive/negative ion primary MS with electrospray ion source and online mass correction with LockSpray™ correction system; scanning detection: m/z 50-1500; scanning time: 0.2 s; capillary voltage: 3.0 kV; cone voltage of sample: 35 V; extraction voltage: 4.0 V; ion source temperature: 120 °C; desolvent temperature: 350 °C; cone gas flow: 50 L/h; desolvent nitrogen flow: 650 L/h; and impact energy: 6-30 V.

Statistical analysis

The data were processed using SPSS version 17.0 software and expressed as mean x ± standard deviation. On the basis of evaluating the variance of the original data in accordance with normal distribution, the differences between groups were compared by single factor ANOVA, and the differences between groups were analyzed by t-test. $P < 0.05$ meant that the difference was statistically significant.

Biomarkers analysis

The determined MS data of urine metabolism profile were obtained by data dimension reduction and MS matrix information using Marker lynx software. Data

were analyzed by unsupervised principal component analysis (PCA) of ssLynxV4.1 workstation software, and PCA score chart was obtained to reflect the clustering degree of each group. Orthogonal partial least squares discriminant analysis (OPLS-DA) was used to obtain the OPLS-DA score chart reflecting the contribution rate of the metabolic contour and to draw the scores plot. The farther the distance points in the load diagram, the greater the contribution of ions to the metabolic contour trajectory. The metabolic group map of each group was analyzed by pattern recognition, and the observed values of each group were distinguished. The measurement data were collected for statistical analysis. Potential biomarkers were screened out based on variable importance in projection (VIP) and VIP confidence interval estimation. Ions of VIP > 1.5 were taken for comparison among groups, and the *t* test was used for inter-group difference. $P < 0.05$ was considered as the screening condition to obtain potential biomarkers. Secondary MS analysis was conducted for the biomarkers. The structural analysis of the biomarkers was carried out based on its relative molecular mass and molecular group structure. Based on the results of the analysis, the biological significance of biomarker information retrieval and the metabolic pathway of biomarkers were analyzed and explained using the human metabolic group database, Scripps Metabolic and Mass Spectrometry Center, Kyoto Encyclopedia of Genes and Genomes and published literature. Thus, the core biomarkers closely related to the occurrence and development of IBS model were screened according to the literature.

RESULTS

Overall characterization of urine metabolic profile

Urine samples of rats on days 0 and 14 were analyzed based on the optimized analysis conditions of UPLC-Q-TOF-MS. In the positive and negative ion scanning mode, the urine samples were scanned and analyzed within a set mass range, and base peak ion of the mass spectrum profile was obtained (Figures 1-4). The urine samples from the different groups had a better differentiation degree and different metabolite spectrum showing better separation and response effect.

Principal component analysis of urine metabolic data

Urine metabolomics study of IBS disease model: The PCA and OPLS-DA score charts are shown in Figure 5 and Figure 6. There was no overlap of data points in urine of IBS model rats compared with that of the normal control group, and the data was obviously classified in a different quadrant. The obvious separation status in score plots suggested that the urine metabolism in two groups was distinguished well. This indicated that there was an obvious difference in metabolites of rats between the normal group and IBS model group. Before and 14 d after medication, the separation in the two score charts was relatively obvious, indicating that the IBS model rats were stable within 14 d.

Urine metabolomics study on intervention of Tong Xie Yao Fang on adaptive IBS disease model: The PCA and OPLS-DA score charts are displayed in Figure 7. Compared with Tong Xie Yao Fang groups, the data points for urine in IBS rats (0 d) have overlap and were clearly classified in the same quadrant, showing unseparated state in the score charts. The metabolites in the model group and Tong Xie Yao Fang groups were the same induction by rectal dilatation and maternal separation stimulation, indicating successful modeling of IBS. Compared with the normal group, the data points for urine in IBS model rats and Tong Xie Yao Fang groups exhibited no overlap and were clearly classified in different quadrants, showing obvious separation state in score plots and well-distinguished well metabolism. This indicated that after modeling of rats, there were obvious differences in metabolites between IBS rats and normal rats, and the metabolite component in the urine showed significant changes.

The PCA and OPLS-DA score plots are shown in Figure 8. The data points for urine in the IBS model rats and Tong Xie Yao Fang groups treated for 14 d were dispersed and clearly classified in different quadrants, showing separation in score plots and distinct urine metabolism. The results illustrate that the metabolic products have obvious changes after treatment with Tong Xie Yao Fang in the IBS model rats compared with the model control group. Tong Xie Yao Fang treatment groups showed separation in score plots, but the degree of differentiation was not obvious. The distribution of data points in the medium-dose and high-dose groups was similar to that of the normal group.

Further research showed that the urine samples of rats in each group showed complete separation status in the 2D space of the PCA score chart under the condition

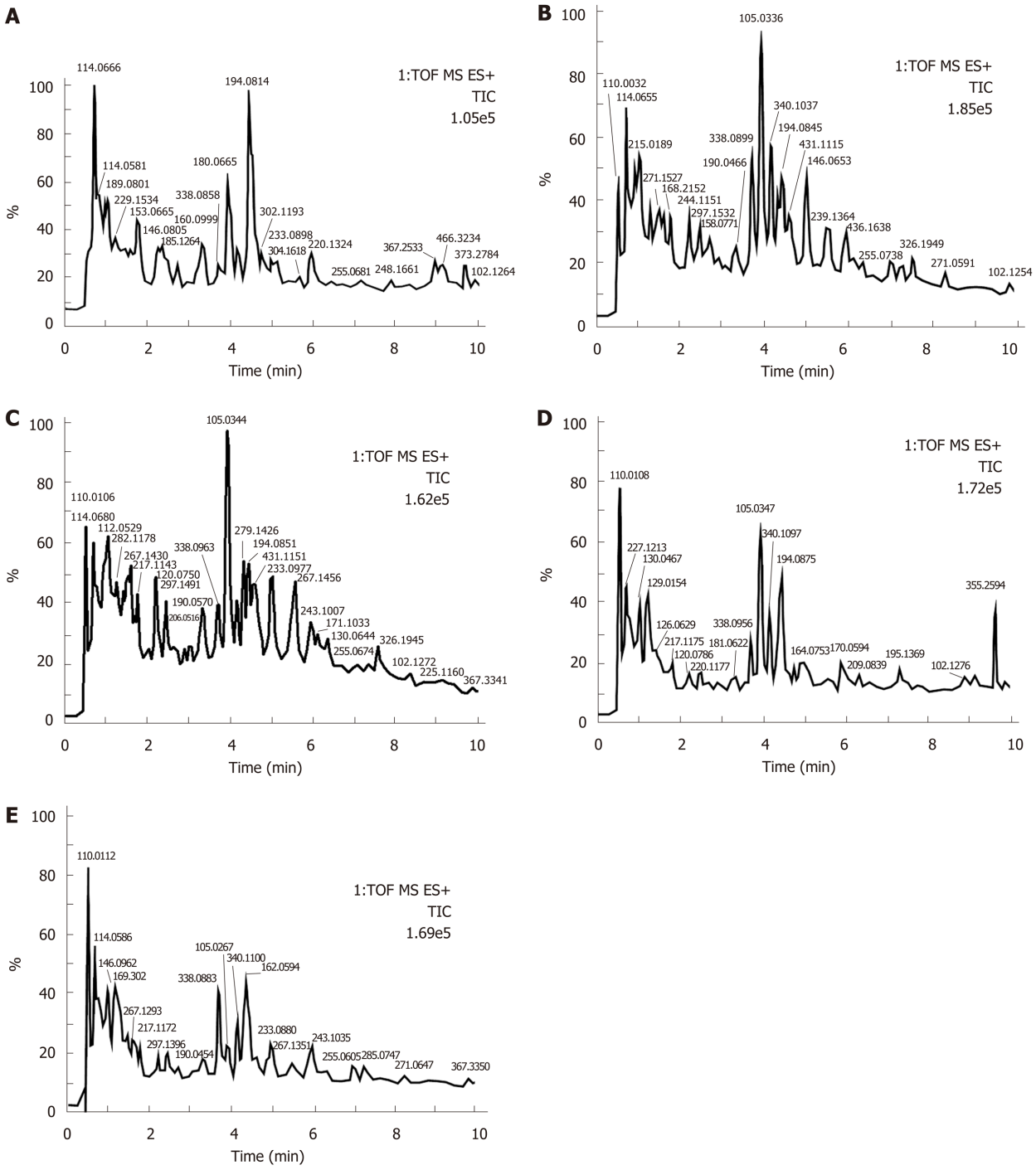

Figure 1 Base peak ion chromatograms in positive ion mode of metabolites in urine of rats treated for 0 d. A: Normal group (K); B: Model control group (M); C: Tong Xie Yao Fang high dose group (G); D: Tong Xie Yao Fang medium dose group (Z); E: Tong Xie Yao Fang low dose group (D).

of positive and negative ions. This indicated that physiological function was changed by rectal distention and maternal separation stimulation. This reflected changes in urine metabolic profile of rats as well as liver stagnation and spleen deficiency syndrome of IBS and metabolic characterization of Tong Xie Yao Fang treatment. The scores chart for evaluating the contribution of the metabolic profile grouping was obtained by analyzing the metabolic profile of samples with OPLS-DA. In addition, pattern recognition analysis was conducted for metabolomics profiles of urine samples. As shown in Figures 5-7, the data points in each group exhibited no/less overlap, indicating that there were differences among the groups. Based on VIP and VIP confidence interval, the potential biomarkers were evaluated.

Identification of potential biomarkers

Potential biomarkers were screened according to VIP, and the differential variables

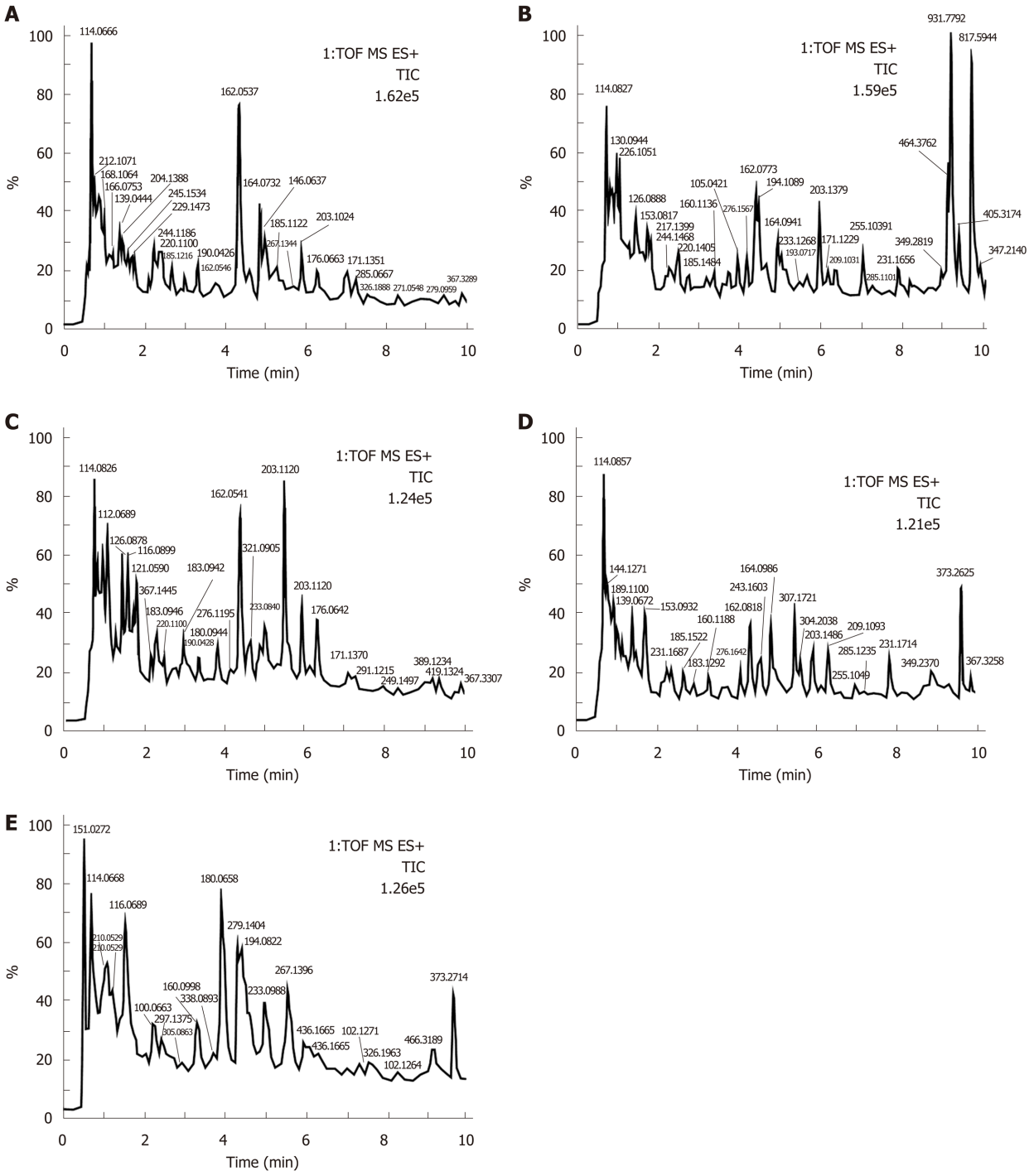

Figure 2 Base peak ion chromatograms in positive ion mode of metabolites in urine of rats treated for 14 d. A: Normal group (K); B: Model control group (M); C: Tong Xie Yao Fang high dose group (G); D: Tong Xie Yao Fang medium dose group (Z); E: Tong Xie Yao Fang low dose group (D).

with VIP > 1.5 were selected. The screened differential variables were verified by Anova, and the differential variables with $P < 0.05$ were considered potential biomarkers.

Based on the obtained identification results of molecular ion fragments, nine kinds of biomarkers in urine were identified through the human metabolic group database and METLIN, which are related to the metabolites of the Tong Xie Yao Fang treating IBS and showing a certain uptrend or downtrend. These biomarkers belong to amino acids, organic acids, succinyl and glycosides. Meanwhile, the metabolic pathways of L-serine, L-threonine and 5-hydroxy-L-tryptophan (5-HTP) can be searched in the Kyoto Encyclopedia of Genes and Genomes (Table 1). These pathways mainly include cysteine and methionine metabolism, vitamin B6 metabolism, serotonin synapse, tryptophan metabolism, sphingolipid metabolism, protein digestion and absorption and amino acid metabolism (Table 1 and Table 2).

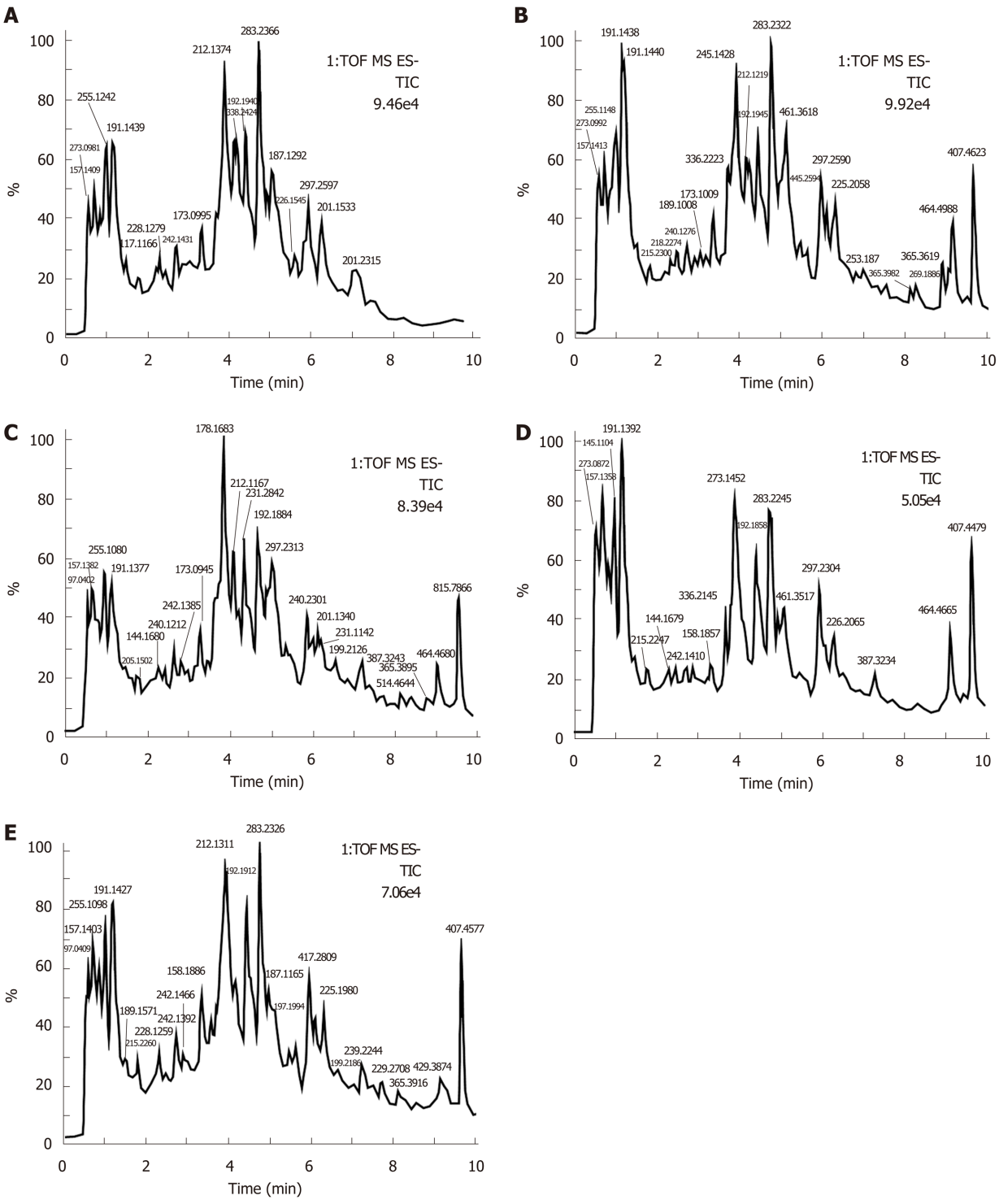

Figure 3 Base peak ion chromatograms in negative ion mode of metabolites in urine of rats treated for 0 d. A: Normal group (K); B: Model control group (M); C: Tong Xie Yao Fang high dose group (G); D: Tong Xie Yao Fang medium dose group (Z); E: Tong Xie Yao Fang low dose group (D).

DISCUSSION

Metabonomics is the systematic scientific study of metabolic product changes and their ability to reveal the metabolic nature of the life activities of an organism. After the effect of changes in external conditions on the overall state of the body, the effect of the external environment on the metabolites is studied by taking all the small molecular substances as the observation target. Metabolomics is an important research method of systems biology, which has the characteristics of measuring overall metabolic dynamics, synthesis and analysis. It studies the metabolic changes in metabolism and metabolites caused by drugs. The changes in endogenous

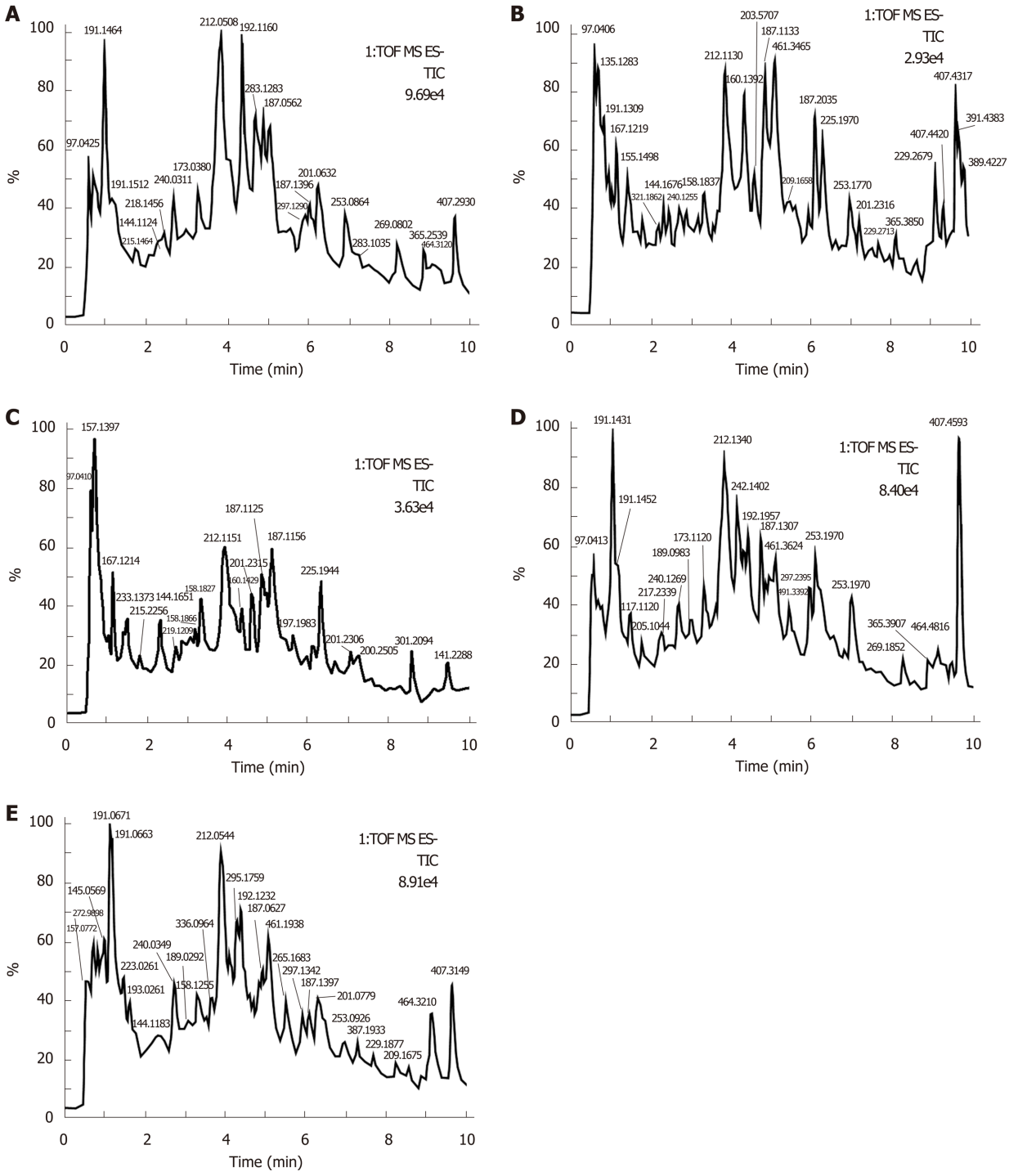

Figure 4 Base peak ion chromatograms in negative ion mode of metabolites in urine of rats treated for 14 d. A: Normal group (K); B: Model control group (M); C: Tong Xie Yao Fang high dose group (G); D: Tong Xie Yao Fang medium dose group (Z); E: Tong Xie Yao Fang low dose group (D).

metabolites can directly reflect the changes in biochemical processes *in vivo*. Drugs have specific effects on certain processes of metabolic pathways involved with the main biomarkers, which causes marked changes in the endogenous metabolites[9]. Metabonomics has the technical characteristics of overall comprehensive information representation and analysis of provenance. By detecting the metabolic fingerprints of body fluids and analyzing the causes of metabolic spectrum changes, the changes in endogenous metabolites induced by drugs can be studied, and the role of Chinese medicines in metabolic regulation and their exact mechanism of action can be explored. Thus, it may clarify the relationship between compound prescriptions and disease syndromes.

Tong Xie Yao Fang was first recorded in "*Danxi Xinfa*" cathartic volume, which is used to treat diarrhea with pain caused by spleen deficiency and liver hyperactivity.

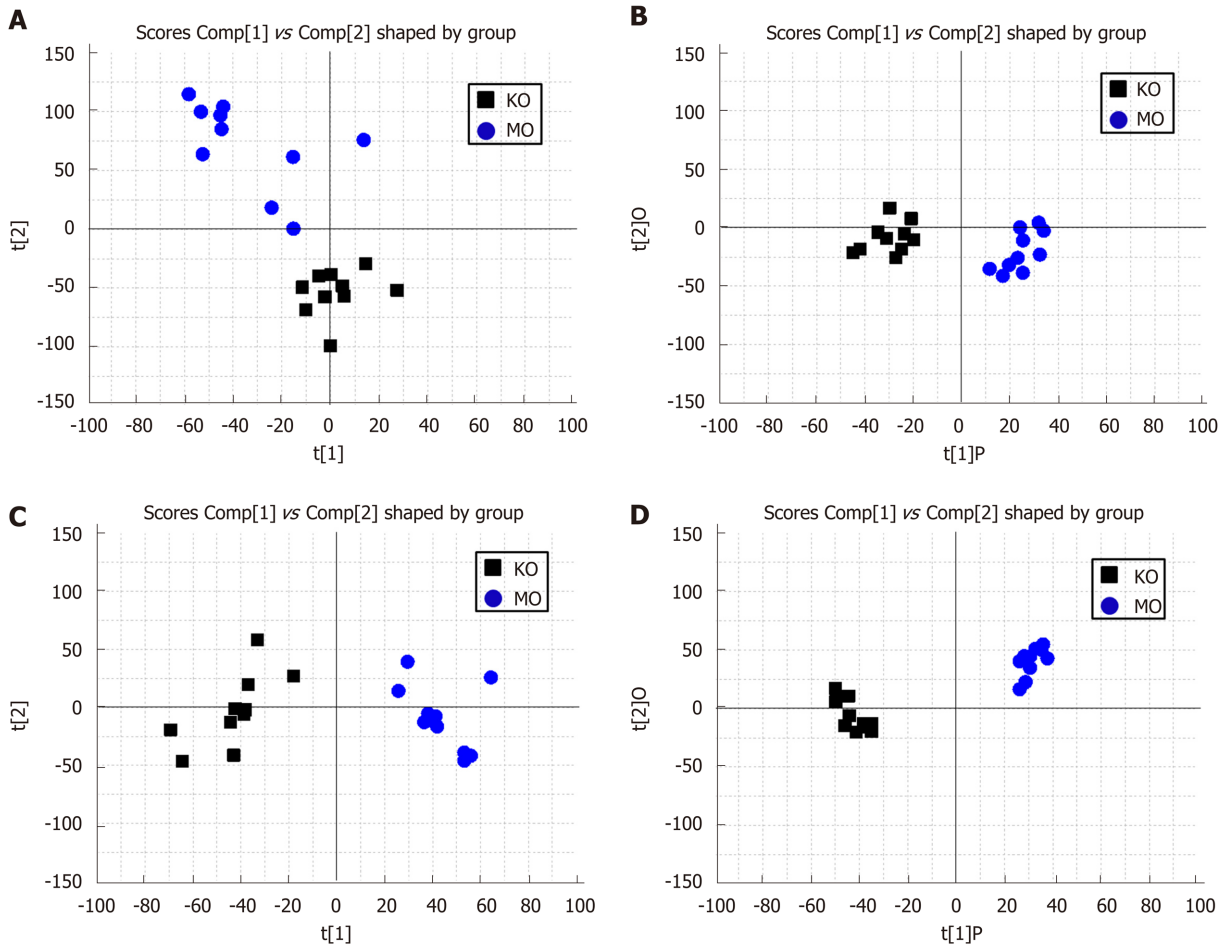

Figure 5 Score plot of metabolites in urine of normal and model rats treated for 0 d. A: Principal component analysis (PCA) score plot (positive ion); B: Orthogonal partial least squares discriminant analysis score plot (positive ion); C: Principal component analysis score plot (negative ion); D: Orthogonal partial least squares discriminant analysis score plot (negative ion). K: Normal group; M: Model control group.

Liver and spleen deficiency syndrome is due to liver drainage, spleen-soil deficiency, transversely invading spleen and spleen transport dereliction of duty. Tong Xie Yao Fang can tone the spleen, clear damp, stop diarrhea, knead liver to soothe qi and relieve pain and make the spleen healthy and liver soft. Then, the pain and diarrhea are resolved.

At present, the rectal distention stimulation of the suckling rat is a widely used method and the most reasonable animal model for IBS, which is a method of local mechanical stimulus. Mother/child separation is an early life event that can simulate emotional anxiety and causes increased stress response and visceral hypersensitivity[13]. Our research group reproduced the IBS model by combining the above two methods. Tong Xie Yao Fang had a significant effect of tonifying the spleen and liver stagnation on the IBS model derived from the combination of rectal distention and mother/child separation of neonatal rats. The model symptoms were spleen deficiency and liver hyperactivity and were related to the prescription of Tong Xie Yao Fang and syndrome.

In this study, the metabonomics of IBS rat urine was studied by UPLC-Q-TOF-MS analysis combined with Mass Lynx software. Compared to the metabolic results of the normal group, the contents of L-serine, succinylacetone, proline-hydroxyproline, valyl-serine, marmesin rutinoside and 5-hydroxy-L-tryptophan (5-HTP) increased while the contents of L-threonine and acetyl citrate decreased in the Tong Xie Yao Fang treatment groups and the model group,. Tong Xie Yao Fang decreased the contents of L-serine, succinylacetone, proline-hydroxyproline, valyl-serine, marmesin rutinoside and 5-HTP and increased the contents of L-threonine and acetyl citrate. The metabolic pathways involved serotonin synapse, tryptophan metabolism, cysteine and methionine metabolism, vitamin B6 metabolism, sphingolipid metabolism and biosynthesis of proteins and amino acids. The metabolic biomarkers may be L-serine, 5-HTP, L-threonine, valyl-serine and acetyl citrate. Therefore, it is speculated that the regulatory mechanism of Tong Xie Yao Fang may involve the following aspects.

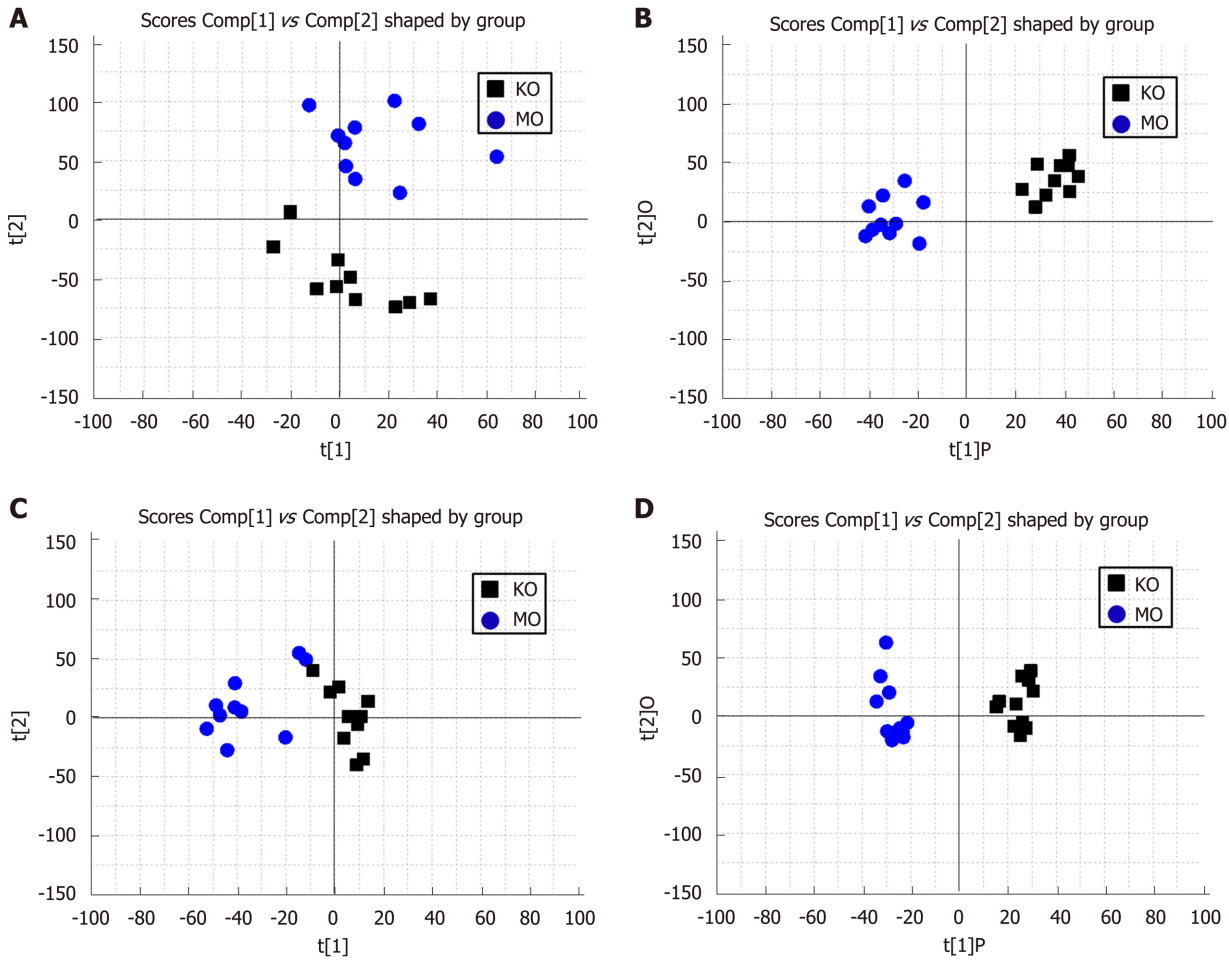

Figure 6 Score plots of metabolites in urine of normal and model rats treated for 14 d. A: Principal component analysis score plot (positive ion); B: Orthogonal partial least squares discriminant analysis score plot (positive ion); C: Principal component analysis score plot (negative ion); D: Orthogonal partial least squares discriminant analysis score plot (negative ion). K: Normal group; M: Model control group.

Serotonin synapse and tryptophan metabolism

Serotonin (5-HT) escapes from presynaptic cells and transmits information to postsynaptic cells, thus acting as a neurotransmitter. 5-HTP is involved in the serotonin synapse pathway and tryptophan metabolism. In this pathway, 5-HTP is primarily involved in serotonin synthesis. 5-HTP is converted from the tryptophan hydroxylase through tryptophan followed by decarboxylation to synthesize 5-HT. 5-HT is a neurotransmitter that widely exists in the nervous system and gastrointestinal tract and participates in the regulation of psychological and neural functions. As a signal transduction molecule, it can be an important neurotransmitter in the brain-gut axis[14]. 5-HT in the digestive tract can enhance the sensitivity of the gastrointestinal splanchnic nerves, regulate the movement of the gastrointestinal tract and participate in the regulation of intestinal sensation, secretion and movement. 5-HT in the central nervous system can cause mental and behavioral disorders and regulate mood and sleep. 5-HT signaling disorder is closely related to IBS, and both physiological and pathological stimuli can lead to an increase in 5-HT release. Studies have shown that the sensory hypersensitivity in IBS patients may be related to increased sensitivity of visceral afferent nerves, increased excitability of spinal dorsal horn neurons and changes in central sensory regulation. The change in 5-HT level is closely related to the occurrence and development of many complex diseases and syndromes, including IBS and plays a dominant role in its pathogenesis. High concentrations of 5-HT can enhance the sensitivity to external stress of visceral afferent nerve endings and the enteric nervous system and then stimulate the release and secretion of various neurotransmitters, stimulate and expand the activity and participate in the biochemical signaling process of brain-gut interaction.

A previous study showed that the content of 5-HT in the brain-gut axis of IBS model rats was significantly higher than that in the normal group, which also confirmed the view that an increase in 5-HT level is an important part of the

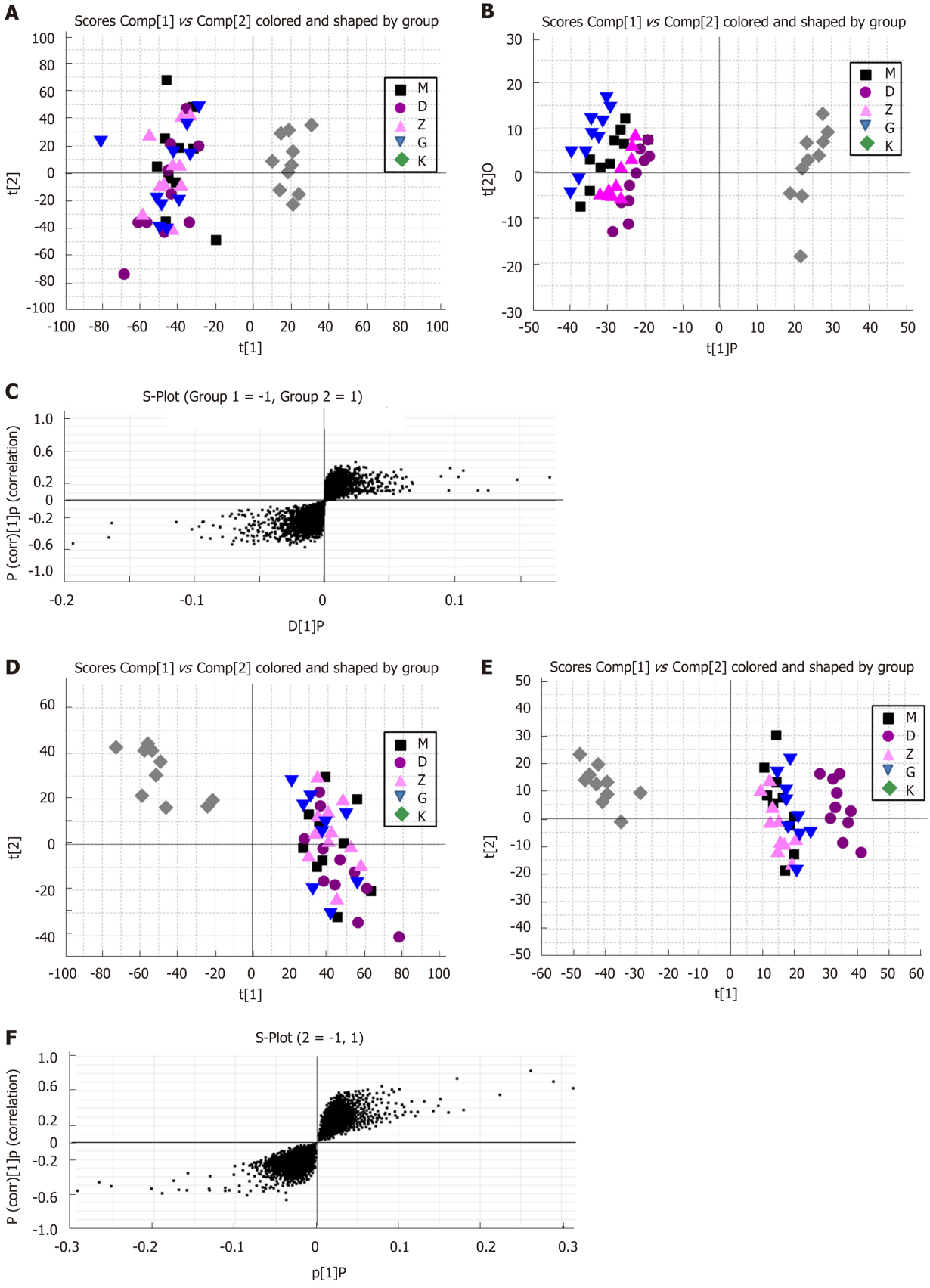

Figure 7 Score plot of metabolites in urine of rats treated for 0 d. A: Urine principal component analysis score plot (positive ion); B: Urine orthogonal partial least squares discriminant analysis score plot (positive ion); C: Urine score plot (positive ion); D: Principal component analysis score plot (negative ion); E: Orthogonal partial least squares discriminant analysis score plot (negative ion); F: Urine score plot (negative ion). K: Normal group; M: Model group; G: Tong Xie Yao Fang high dose group; Z: Tong Xie Yao Fang medium dose group; D: Tong Xie Yao Fang low dose group.

pathogenesis of IBS. The increase of 5-HTP metabolism in the urine of IBS model rats

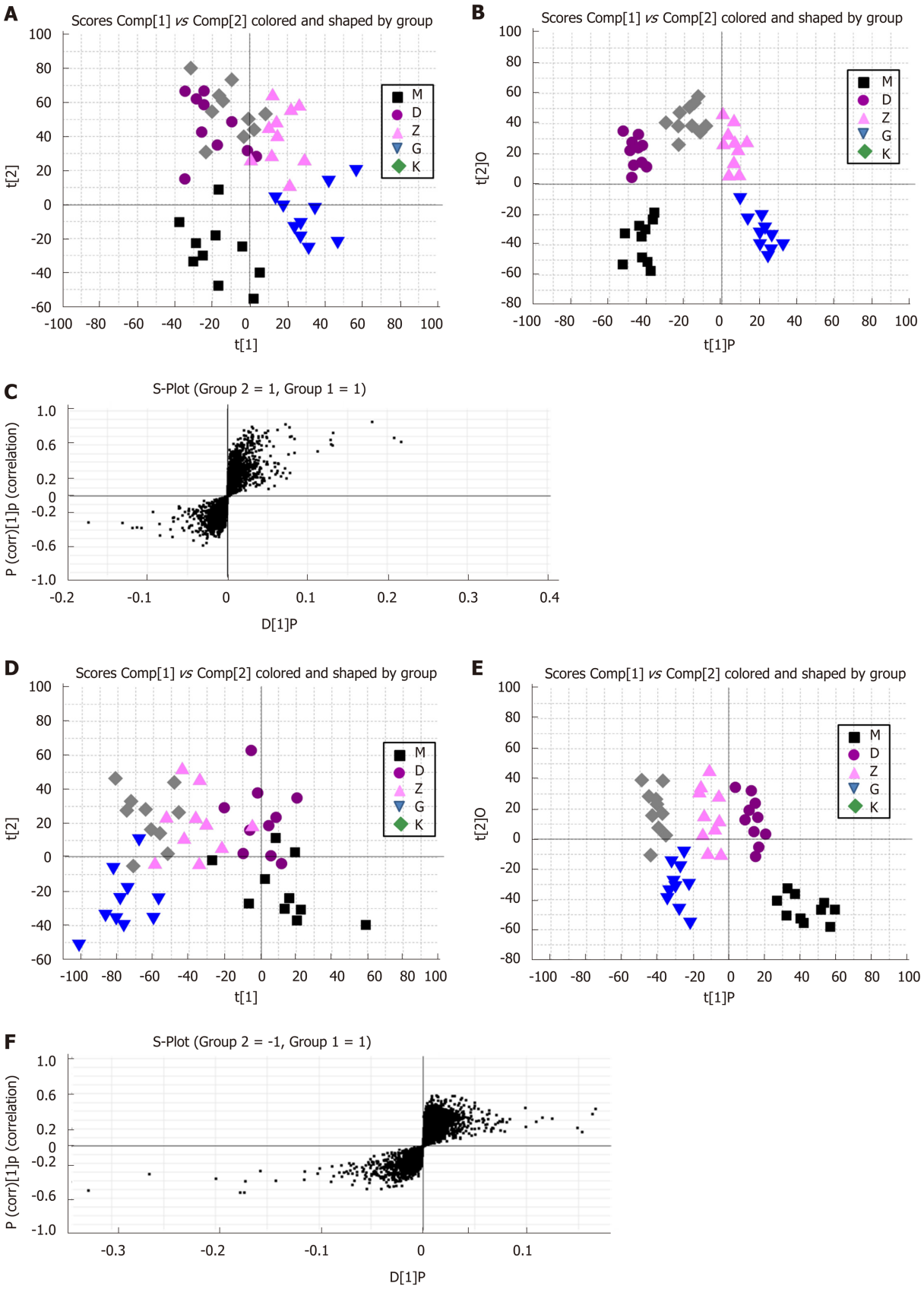

Figure 8 Score plot of metabolites in urine of rats treated for 14 d. A: Urine principal component analysis score plot (positive ion); B: Urine orthogonal partial least squares discriminant analysis score plot (positive ion); C: Urine score plot (positive ion); D: Principal component analysis score plot (negative ion); E: Orthogonal partial least squares discriminant analysis score plot (negative ion); F: Urine score plot (negative ion). K: Normal group; M: Model control group; G: Tong Xie Yao Fang high dose group; Z: Tong Xie Yao Fang medium dose group; D: Tong Xie Yao Fang low dose group.

indicates that there are pathological mechanisms of abnormal secretion, release and

Table 1 Information of potential biomarkers in urine of irritable bowel syndrome model

No.	HMDB ID	Retention time/min	M/Z	Chemical formula	VIP	Select the ions	Common name	Expression trend			
								M *vs* K	G *vs* M	Z *vs* M	D *vs* M
1	HMDB00187	3.854	105.04	$C_3H_7NO_3$	2.00494	$[M + H]^+$	L-serine	↑	↓	↓	↓
2	HMDB13198	8.663	184.04	$C_8H_8O_5$	4.85675	$[M-H]^+$	4-methylgallic acid	↑	↓	↓	↓
3	HMDB00167	3.204	119.06	$C_4H_9NO_3$	3.76891	$[M + H]^-$	L-threonine	↓	↑	↑	↑
4	HMDB00635	2.915	158.06	$C_7H_{10}O_4$	8.66271	$[M-H]^-$	Succinylacetone	↑	↓	↓	↓
5	HMDB29018	1.084	228.11	$C_{10}H_{16}N_2O_4$	5.22312	$[M + H]^+$	Prolyl-hydroxyproline	↑	↓	↓	↓
6	HMDB29136	4.445	204.11	$C_8H_{16}N_2O_4$	4.11234	$[M + H]^-$	Valyl-serine	↓	↑	↑	↑
7	HMDB59808	4.954	232.02	$C_8H_8O_8$	2.23843	$[M + H]^+$	Acetyl citrate	↓	↑	↑	↑
8	HMDB41413	7.827	554.20	C26H24O13	9.34210	$[M + H]^+$	Marmesin rutinoside	↑	↓	↓	↓
9	HMDB00472	1.213	220.08	$C_{11}H_{12}N_2O_3$	5.13710	$[M + H]^-$	5-hydroxy-L-tryptophan	↑	↓	↓	↓

VIP: Variable importance in projection; HMDB: Human metabolic group database.

metabolism of 5-HT in the IBS model, which is consistent with previous studies[15]. Tong Xie Yao Fang can reduce the 5-HTP content in urine. Tong Xie Yao Fang may be involved in the regulation of IBS visceral hypersensitivity and gastrointestinal motility by regulating 5-HT metabolism and synapses to relieve IBS in model rats. It is suggested that the improvement of spleen deficiency and liver hyperactivity of IBS by Tong Xie Yao Fang is related to the regulation of the serotonin synaptic pathway and tryptophan metabolism.

Cysteine and methionine metabolism

Halfcystine (Hcy) is synthesized from methionine and L-serine *via* cystathionine, which is the intermediate product of the cysteine and methionine metabolism. Hcy has high biological activity and is involved in a variety of inflammatory and immune disorders. Studies have shown that mild inflammation and immune activation of intestinal mucosa also play a key role in the pathogenesis of IBS. Hcy is closely related to IBS inflammatory immune pathogenesis[16]. Impaired intestinal barrier function plays an important role in the pathogenesis of IBS. Hcy can produce a large number of oxidative free radicals that damage the intestinal mucosal mechanical barrier[17] and changes intestinal permeability, leading to massive invasion of inflammatory factors and aggravation of IBS pathophysiology[18]. Inflammatory factors such as Hcy and tumor necrosis factor-α (TNF-α) promote each other in the process of inflammatory immunity, aggravating the inflammatory response[19].

In addition, the content of Hcy is positively correlated with the incidence of depression. IBS patients are often accompanied by depression, anxiety and other psychological disorders. The hypothesis of the pathophysiology of the biological-psychological- sociological model of IBS emphasizes the importance of emotional disturbance in the pathogenesis of IBS[20]. Psychological stress may alter systemic and intestinal immunity. The dispose of central pivot on mental disorders and stimulation of visceral injury may involve brain-gut axis dysfunction. Experimental studies have shown that the consumption of sugar and water and the open field test were improved in rats after the intervention of Hcy metabolic coenzyme, indicating that the level of Hcy is related to anxiety-like behavior or depression-like behavior in IBS rats. The content of Hcy decreases and anxiety-like behavior or depression-like behavior is also reduced[16]. Therefore, it can be inferred that Hcy is closely related to not only IBS inflammatory immune response but also psychological stress such as IBS-related depression.

As a key substrate for the metabolism of sulfur transfer in Hcy, L-serine content increases in the urine of IBS model rats, suggesting abnormality of the metabolic pathway of Hcy sulfur transfer in IBS rats. The abnormal metabolism of L-serine in the process of IBS indicates that L-serine is involved in the pathophysiological

Table 2 Information of metabolic pathways involved in urine biomarkers in irritable bowel syndrome model rats referred to Kyoto Encyclopedia of Genes and Genomes

No.	Common name	Metabolic pathway ID	Metabolic pathway name
1	L-serine	map00270	Cysteine and methionine metabolism
		map00600	Sphingolipid metabolism
		map04974, map01230, map00260	Protein digestion and absorption; biosynthesis of amino acid; glycine, serine and threonine metabolism
2	L-threonine	map00750	Vitamin B6 metabolism
		map04974, map01230, map00260	Protein digestion and absorption; biosynthesis of amino acid; glycine, serine and threonine metabolism
3	5-hydroxy-L-tryptophan	map04726	Serotonergic synapse
		map00380	Tryptophan metabolism

process. It is suggested that Tong Xie Yao Fang regulates cysteine and methionine metabolism through L-serine and improves the intestinal reaction and emotional changes of IBS. Tong Xie Yao Fang improves liver hyperactivity syndromes such as emotional depression, irritability, abdominal pain, abdominal distension and borborygmus of IBS rats, which is related to regulation of the cysteine and methionine metabolism pathways embodying the effects of "restricting" the liver.

Vitamin B6 metabolism

L-threonine is involved in the metabolism of vitamin B6. O-phospho- 4-hydroxy-L-threonine can be converted to 4-hydroxy-L-threonine by the enzyme to form pyridoxine, and pyridoxal and pyridoxamine can be transformed into each other. Vitamin B6 is involved in the formation of serotonin, which can increase the serotonin concentration. Additionally, vitamin B6 as a coenzyme participates in various metabolic reactions such as amino acids and proteins. Lack of vitamin B6 can lead to the stagnation of protein hydrolysis and conversion to fat, thus stopping growth. The level of L-threonine amount increases after taking Tong Xie Yao Fang, enhancing vitamin B6 synthesis, increasing the serotonin content and promoting the protein metabolism.

Sphingolipid metabolism

L-serine is involved in the synthesis of sphingolipids. Sphingolipids and their metabolites play an important role in maintaining cell growth and signal transduction. Ceramide is the simplest structure of sphingolipid, the precursor of synthetic complex sphingolipid, which is an intermediate in the metabolic and transformation pathway[21]. Sphingolipids can regulate immune function and inflammatory response. TNF-α can directly result in the production and release of ceramide from mitochondria[22]. It has been demonstrated that the clinical symptoms of IBS are associated with low inflammatory response. Many researchers have reported that TNF-α plays a major role in the development of IBS inflammatory immune response[23,24]. TNF-α is a pro-inflammatory cytokine with various biological activities. It interacts with other cytokines to induce or increase the production of inflammatory mediators and participates in the development of inflammatory immune responses. The level of L-serine decreases after treatment with Tong Xie Yao Fang, which indicates that the content of ceramide synthesized by L-serine is reduced, and the inflammatory immune response of IBS is alleviated. The mechanism may be related to the reduction of pro-inflammatory factor TNF-α involved in the inflammatory immune response.

Metabolism of protein and amino acids

L-serine and L-threonine are also involved in the biosynthesis of proteins, amino acids and sugars. Energy metabolism is the release, transfer and utilization of energy produced in metabolic processes and is closely related to the theory of "spleen governing movement transformation" that is an important part of the spleen-stomach doctrines in TCM. L-threonine can promote growth and improve immune function. Serine plays a role in the metabolism of fats and fatty acids, the growth of muscles and maintains the immune system. As a tricarboxylic acid substrate, acetyl citrate participates in the tricarboxylic acid cycle as a source of energy. It is suggested that IBS rats have the disturbance of energy metabolism, immune dysfunction and growth inhibition, which are characteristics of spleen deficiency syndrome in TCM. In

addition, L-serine and L-threonine are involved in amino acid metabolism. Tong Xie Yao Fang can increase or decrease the related metabolites and make the metabolic network return to normal or improve, indicating that Tong Xie Yao Fang can improve the energy metabolism from sugar and amino acid metabolism in IBS spleen deficiency syndrome. These results suggest that Tong Xie Yao Fang can improve the body spleen deficiency and other spleen deficiency syndromes in IBS rats, enhance energy metabolism and regulate immune function, which reflects the function of tonifying spleen.

In summary, endogenous metabolites evidently change after the replication of IBS model with Tong Xie Yao Fang treatment. The identified metabolic pathways include serotonin synapse and tryptophan metabolism, cysteine and methionine metabolism, vitamin B6 metabolism, sphingolipid metabolism and biosynthesis of proteins, amino acids and sugars. Due to the complexity of regulation, we do not fully understand the biological significance of abnormal expression of endogenous metabolites. However, the results suggest that the abnormal characteristics of metabolites might form the biological basis of liver hyperactivity and spleen-deficiency syndrome in IBS models. Tong Xie Yao Fang plays a complex role in regulating endogenous metabolites related to the syndrome of liver and spleen deficiency, representing the effects of supporting the spleen and restricting the liver.

ARTICLE HIGHLIGHTS

Research background

The establishment of disease and syndrome combined with animal model in traditional Chinese medicine (TCM) is an important link to realize the objectiveness, standardization and scientificity of TCM. At present, the model construction method of the disease and syndrome combination for irritable bowel syndrome (IBS) is mainly based on the modern medical disease animal model combined with syndrome related etiology and disease mechanism. There are obvious differences in metabolic products among TCM syndromes. Metabonomics finds biomarkers of diseases by comparing the metabolic profiles of animal body fluids, analyzing the differences of metabolic pathways and understanding the pathological process and metabolic pathways of substances in the body. Thus, the evaluation of animal models tends to be comprehensive and objective. Therefore, through the combination of metabonomics and other systems biology with TCM prescription syndrome, we can clearly define the attributes of animal model syndrome. Through identical formulas, different models and different formulas, we can determine the model of syndrome from the perspective of metabolic products *in vivo*, clarify the mechanism and essence of prescriptions and drugs and clarify the correlation between prescriptions and syndromes. This research idea not only conforms to the research requirements of TCM prescriptions and syndromes but also follows the regularity between phenomena and essence in modern science.

Research motivation

Tong Xie Yao Fang is effective in the treatment of IBS. The overall concept of TCM theory and the treatment of evidence differentiation require that the clinical differentiation of IBS must be accurate. Therefore, the establishment of a syndrome-binding IBS model is the key to research of IBS in TCM. In recent years, the study of disease syndrome combined with animal model has developed rapidly and has become a new direction of animal model development in TCM research. At present, many scholars have provided a suitable combination model of disease and syndrome for the study of TCM in the treatment of IBS, which reflects the advantages of TCM on IBS syndrome differentiation. Therefore, in the aspect of disease and syndrome combination model replication, it is closely related to the advanced research methods of modern medicine. Through the combination of metabonomics and other system biology with TCM prescription syndrome, the attributes of IBS disease syndrome combined with animal model syndrome can be clearly defined. Through the improvement of the construction method and train of thought of the disease syndrome model, a more mature disease syndrome combined with animal model would be obtained, and the scientific connotation of TCM will be expounded.

Research objectives

The research objective is to discover the biomarkers of Tong Xie Yao Fang and establish its metabolic model characteristics through its influence on metabolites of IBS. The action essence of Tong Xie Yao Fang and the syndrome essence of its disease model are explored based on the changing law of substance quality *in vivo*.

Research methods

In this study, Wistar rats were used to establish the IBS models, and then randomly divided into four groups: Model control group, Tong Xie Yao Fang treatment groups (high, medium, low doses). A normal, non-IBS group was established. The rats were treated for 2 wk. On days 0 and 14 of the experimental model, urine was collected for 12 h and was analyzed by ultra-performance liquid chromatography quadrupole time-of-flight mass spectrometry. Nine potential biomarkers were identified, and six major metabolic pathways were found to be related to IBS.

Research results

In the study of metabonomics, nine potential biomarkers including L-serine, 4-methylgallic acid, L-threonine, succinylacetone, prolyl-hydroxyproline, valyl-serine, acetyl citrate, marmesin rutinoside and 5-hydroxy-L-tryptophan were identified in urine, which were assigned to amino acids, organic acids, succinyl and glycosides. Furthermore, the metabolic pathway of L-serine, L-threonine and 5-hydroxy-L-tryptophan was found in the Kyoto Encyclopedia of Genes and Genomes, which mainly involved the metabolism of cysteine and methionine, vitamin B6 metabolism, serotonin synapse, tryptophan metabolism, sphingolipid metabolism, digestion and absorption of protein and amino acid metabolism. These pathways are related to intestinal dysfunction, inflammatory syndrome, nervous system dysfunction and other diseases. However, due to the complexity of regulation, we do not fully understand the biological significance of abnormal expression of endogenous metabolites.

Research conclusions

The endogenous metabolites changed significantly for the IBS model after the intervention of Tong Xie Yao Fang. Tong Xie Yao Fang plays a complex regulatory role in endogenous metabolites associated with liver spleen deficiency syndrome. It is suggested that the improvement of spleen deficiency and liver hyperactivity of IBS by Tong Xie Yao Fang is related to the regulation of the serotonin synaptic pathway and tryptophan metabolism. It was confirmed that the rectal dilatation stimulation combined with maternal-infant separation of IBS animal model was the best model for the adaptation of Tong Xie Yao Fang. In this study, through metabonomics related research on Tong Xie Yao Fang, endogenous metabolites in animal models were evident, suggesting the importance of syndrome differentiation and treatment of clinical syndrome in TCM.

Research perspectives

Metabonomics, genomics, transcriptome, and proteomics have the characteristics of systematic methodology such as overall dynamics, synthesis and analysis and are similar to the overall concept of TCM in the treatment of diseases. By means of systematic biology research method, prescriptions and syndromes can be integrated into an organic system for research. Therefore, the subject not only meets the research requirements of prescriptions and syndromes in TCM but also conforms to the regularity of the relationship between phenomena and essence in modern science.

REFERENCES

1 **Bian Z**, Wu T, Liu L, Miao J, Wong H, Song L, Sung JJ. Effectiveness of the Chinese herbal formula TongXieYaoFang for irritable bowel syndrome: a systematic review. *J Altern Complement Med* 2006; **12**: 401-407 [PMID: 16722791 DOI: 10.1089/acm.2006.12.401]

2 **Yin Y**, Zhong L, Wang JW, Zhao XY, Zhao WJ, Kuang HX. Tong Xie Yao Fang relieves irritable bowel syndrome in rats via mechanisms involving regulation of 5-hydroxytryptamine and substance P. *World J Gastroenterol* 2015; **21**: 4536-4546 [PMID: 25914462 DOI: 10.3748/wjg.v21.i15.4536]

3 **Xiong XJ**, Wang J, Wang SH, He QY, Tang YL, Fu YK. A Survey of the theoretical Research on the relationship between prescriptions and Diseases Syndromes in Traditional Chinese Medicine. *Zhonghua Zhongyiyao Zaizhi* 2009; **24**: 1624-1626

4 **Xu SJ**, Ma YG, Yang FL. Thoughts and methods of Research on prescription and Syndromes of traditional Chinese Medicine. *Sichuan Zhongyi Zazhi* 2007; **25**: 16-18 [DOI: 10.3969/j.issn.1000-3649.2007.06.009]

5 **Fan X**, Wang J, Jiang YW, Tao CH, Liu SL, Li JG. Primary study on chronic heart failure rats with Yang deficiency syndrome based on theory of correspondence of prescriptions with syndromes. *Zhonghua Zhongyiyao Zazhi* 2015; **30**: 4275-4279

6 **Chen J**, Deng J, Zhou J, Xie ZJ, Gan J, Wen CP. Metabonomics of Artemisia annua and soft-shelled turtle A in combination treatment of systemic lupus erythematosus in mice. *Zhongguo Yaolixue Tongbao* 2016; **32**: 727-732 [DOI: 10.3969/j.issn.1001-1978.2016.05.026]

7 **Wang TS**, Xie M. Metabonomics and Modern Study of Traditional Chinese Medicine. *Zhongyi Zazhi* 2006; **47**: 723-725 [DOI: 10.13288/j.11-2166/r.2006.10.001]

8 **Jing L**, He W, Liu SM, Huang ZH, Liu DM, Zu JX. Research on the effect of Coptis chinensis - Scutellaria baicalensis and Coptis chinensis. Scutellaria baicalensis-Cortex Phellodendri for the 2,4-dinitrophenol-induced fever syndrome model on metabolomics. *Zhongyao Yaoli Yu Linchuang* 2011; **27**: 67-71 [DOI: 10.13412/j.cnki.zyyl.2011.04.004]

9 **Zhou HG**, Chen HB, Wang RP, Zhang Y, Wu MH. Metabonomics and its application in TCM formula study. *Zhongguo Yaolixue Tongbao* 2013; **29**: 161-165 [DOI: 10.3969/j.issn.1001-1978.2013.02.04]

10 **Deng X**. Application of Metabonomics in the Research of traditional Chinese Medicine. *Yunnan Zhongyi Zhongyao Zazhi* 2010; **31**: 73-75 [DOI: 10.16254/j.cnki.53-1120/r.2010.09.020]

11 **Al-Chaer ED**, Kawasaki M, Pasricha PJ. A new model of chronic visceral hypersensitivity in adult rats induced by colon irritation during postnatal development. *Gastroenterology* 2000; **119**: 1276-1285 [PMID: 11054385 DOI: 10.1053/gast.2000.19576]

12 **Larauche M**, Mulak A, Taché Y. Stress-related alterations of visceral sensation: animal models for irritable bowel syndrome study. *J Neurogastroenterol Motil* 2011; **17**: 213-234 [PMID: 21860814 DOI: 10.5056/jnm.2011.17.3.213]

13 **Bian ZX**, Qin HY, Tian SL, Qi SD. Combined effect of early life stress and acute stress on colonic sensory and motor responses through serotonin pathways: differences between proximal and distal colon in rats. *Stress* 2011; **14**: 448-458 [PMID: 21438781 DOI: 10.3109/10253890.2011.558604]

14 **Lu M**, Zhang W, Yao Q, Lu XM, Li SL, Ju JM. Effect of Changkang Fang on serotonin transporter expression in brain-gut axis of visceral hypersensitive rats with irritable bowel syndrome. *Shijie Huaren Xiaohua Zazhi* 2015; **23**: 1231-1237 [DOI: 10.11569/wcjd.v23.i8.1231]

15 **Wang JW**, Xu FH, Yin Y, Zhang JY, Kuang HX. Effects of Tong Xie Yao Fang Formula on multiple factors-induced rat model of irritable bowel syndrome based on the theory of correlation between formula and syndrome. *Zhonghua Zhongyiyao Zazhi* 2017; **32**: 553-559

16 **Zhao Y**, Qian L. Homocysteine-mediated intestinal epithelial barrier dysfunction in the rat model of irritable bowel syndrome caused by maternal separation. *Acta Biochim Biophys Sin (Shanghai)* 2014; **46**: 917-919 [PMID: 25187412 DOI: 10.1093/abbs/gmu076]

17 **Zhang ZZ**, Wang Y, Wang L, Lin KR. Prevention and treatment of intestinal barrier dysfunction by N-acetylcysteine. *Linchuang Junyi Zazhi* 2007; **35**: 756-759 [DOI: 10.3969/j.issn.1671-3826.2007.05.048]

18 **Danese S**, Sgambato A, Papa A, Scaldaferri F, Pola R, Sans M, Lovecchio M, Gasbarrini G, Cittadini A, Gasbarrini A. Homocysteine triggers mucosal microvascular activation in inflammatory bowel disease. *Am J Gastroenterol* 2005; **100**: 886-895 [PMID: 15784037 DOI: 10.1111/j.1572-0241.2005.41469.x]

19 **Yang RX**, Zhang JD, Luo WM, Guo RC, Zhu YY, Wang SH. Effects of"YiShen HuoXue Capsule"on Hcy, ET, TNF-α and NO in Patients with Unstable Angina. *Shanghai Zhongyiyao Zazhi* 2006; **40**: 22-24 [DOI: 10.16305/j.1007-1334.2006.01.009]

20 **Wu JC**. Psychological Co-morbidity in Functional Gastrointestinal Disorders: Epidemiology, Mechanisms and Management. *J Neurogastroenterol Motil* 2012; **18**: 13-18 [PMID: 22323984 DOI: 10.5056/jnm.2012.18.1.13]

21 **Munoz-Olaya JM**, Matabosch X, Bedia C, Egido-Gabás M, Casas J, Llebaria A, Delgado A, Fabriàs G. Synthesis and biological activity of a novel inhibitor of dihydroceramide desaturase. *ChemMedChem* 2008; **3**: 946-953 [PMID: 18236489 DOI: 10.1002/cmdc.200700325]

22 **Bionda C**, Portoukalian J, Schmitt D, Rodriguez-Lafrasse C, Ardail D. Subcellular compartmentalization of ceramide metabolism: MAM (mitochondria-associated membrane) and/or mitochondria? *Biochem J* 2004; **382**: 527-533 [PMID: 15144238 DOI: 10.1042/BJ20031819]

23 **Zhuang LL**, Hu TM, Huang SF, Huang GX. Effects of Huangshu enema on serum levels of TNF-α in patients with irritable bowel syndrome. *Shijie Huaren Xiaohua Zazhi* 2014; **22**: 144-148 [DOI: 10.11569/wcjd.v22.i1.144]

24 **Xiang FM**, Jiang YP, Deng KH. Effect of ShengQing JiangZhuo herbs on the level of TNF-α and IL-10 in serum of patients with IBS. *Zhongguo Zhongxiyi Jiehe Xiaohua Zazhi* 2014; **22**: 638-639, 643 [DOI: 10.3969/j.issn.1671-038x.2014.11.02]

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ORIGINAL ARTICLE

Case Control Study

Association of *XPG* rs2094258 polymorphism with gastric cancer prognosis

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Abstract

BACKGROUND

The xeroderma pigmentosum group G (*XPG*) gene at chromosome 13q33 consists of 15 exons, which may be related to the occurrence and development of gastric cancer (GC).

AIM

To examine the association of several common single nucleotide polymorphisms (SNPs) of the *XPG* gene with GC risk and survival.

METHODS

Five SNPs of *XPG* (rs2094258, rs751402, rs873601, rs2296147, and rs1047768) were genotyped by PCR restriction fragment length polymorphism in 956 histologically confirmed GC cases and 1012 controls in North China. GC patients were followed for survival status and, if deceased, cause of death. Logistic regression and Cox regression were used for analysing associations of *XPG* SNPs with risk of GC and prognosis, respectively. For rs2094258, heterozygous model (CT *vs* CC), homozygous model (TT *vs* CC), recessive model (TT *vs* CT + CC), and dominant model (TT + CT *vs* CC) were analyzed.

RESULTS

None of the examined loci were statistically associated with GC risk, although rs2296147 was marginally associated with GC risk ($P = 0.050$). GC patients with the rs2094258 CT + CC genotype showed worse survival than those with the TT genotype (log-rank test, $P = 0.028$), and patients with the CC genotype had a tendency of unfavourable prognosis compared with those with the TT + CT genotype (log-rank test, $P = 0.039$). The increase in C alleles of rs2094258 [hazard

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ratio (HR) = 1.19, 95% confidence interval (CI): 1.02-1.45, P = 0.037] were associated with the long-term survival of GC cases. Other risk factors for survival included tumor differentiation (HR = 4.51, 95%CI: 1.99-8.23, P < 0.001), lymphovascular invasion (HR = 1.97, 95%CI: 1.44-3.01, P < 0.001), and use of chemotherapy (HR = 0.81, 95%CI: 0.63-0.98, P = 0.041).

CONCLUSION
The *XPG* rs2094258 polymorphism may be associated with overall survival in GC patients.

Key words: Xeroderma pigmentosum group G; Single nucleotide polymorphisms; rs2094258; Gastric cancer; Prognosis

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Core tip: This study investigated the relationships between five functional single nucleotide polymorphisms of the xeroderma pigmentosum group G (*XPG*) (rs2094258, rs751402, rs2296147, rs1047768, and rs873601) and gastric cancer (GC) risk and survival. The results showed an association between the *XPG* rs2094258 polymorphism and overall survival in patients with GC. GC patients with the rs2094258 CT + CC genotype showed a worse survival than those with the TT genotype, and patients with the CC genotype had a tendency of unfavourable prognosis compared with those with the TT + CT genotype. The increase in the number of C alleles of rs2094258 was associated with the long-term survival of GC cases.

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INTRODUCTION

Gastric cancer (GC) is one of the global leading causes of cancer-related death[1]. The World Health Organization (WHO) estimates that 1.03 million people are diagnosed and 783000 die of the disease each year[2]. The highest age standard incidence per 100000 population is observed in Eastern Asia[3], especially in China, at 22.7 per 100000[4]. Multiple factors contribute to the development of this disease, including environmental and genetic factors, such as *Helicobacter pylori* (*H. pylori*) infection, drinking, obesity, a high-salt diet, and various genetic factors[5]. Although GC morbidity and mortality have declined in recent years, the social and economic burden of the disease remains high[1,6,7].

DNA repair genes play a key role in maintaining the genomic DNA stability and integrity. Functional genetic variants of DNA repair genes may change the host DNA repair ability and thus affect tumor prognosis[8]. DNA repair genes participate in many crucial pathways including nucleotide excision repair (NER), which is involved in the repair of some types of DNA damage. Recent evidence suggests that NER factors function in processes that facilitate mRNA synthesis or shape three-dimensional chromatin structure[9]. Xeroderma pigmentosum group G (XPG or ERCC5) plays a key role in NER repair, as it can recognize DNA damage and initiate the NER process[10]. XPG has 3′-junction cutting ability on bubble substrates, resulting in 3′-incision in the human double-incision) repair system, and non-catalytically XPG is needed for subsequent 5′-incision by XPF-ERCC1[11].

Some studies have demonstrated that the single nucleotide polymorphisms (SNPs) of *XPG* may affect the development of cancer, such as lung cancer[12,13], colorectal cancer[14,15], breast cancer[16], neuroblastoma[17], Hodgkin's lymphoma[18], and oral squamous cell carcinoma[19]. However, only a few studies have explored the associations between *XPG* gene SNPs and GC, and those studies show inconsistent results. Some studies reported no associations[20-22], while others demonstrated varying degrees of association[23,24]. Furthermore, few studies have explored the potential prognostic importance of *XPG* SNPs in GC patients[25]. Therefore, we examined GC

risk and survival in relation to common functional *XPG* SNPs in a case-control study in North China.

MATERIALS AND METHODS

Study subjects

From September 2010 to June 2013, pathologically confirmed incident GC cases were selected from the First Affiliated Hospital of Xi'an Jiaotong University. During the same time period, controls were recruited from the Physical Examination Centre of the First Affiliated Hospital of Xi'an Jiaotong University. The cases and controls were matched by sex, age (within 5 years), and residential district. Socio-demographic and clinical data were collected during recruitment, such as alcohol consumption and smoking status. TNM staging of GC tumors was done according to the WHO standard. *H. pylori* infection status was tested by ELISA. The present study protocol was approved by the Institutional Review Board of Health Science Center of Xi'an Jiaotong University. Informed consent was obtained from all study participants.

Follow-up

Cases were followed for survival status and chemotherapy data every 3 mo within the first year and then annually afterwards. Causes and dates of death were recorded, and survival time was calculated from the date of recruitment. Survival time was calculated from time of recruitment to date of death, date of last contact (for those lost to follow-up), or to the last contact with living subjects at the end of the study.

Genotyping

Peripheral blood samples from all cases and controls were collected by the investigators. The TIANamp Blood DNA Kit (Tiangen, Beijing, China) was used for DNA extraction. XPG rs2094258, rs751402, rs873601, rs2296147, and rs1047768 polymorphism genotyping was performed by PCR restriction fragment length polymorphism (PCR-RFLP). The conditions of PCR amplification were: (1) Denaturation at 95 °C for 5 min; (2) 30 cycles of denaturation at 94 °C for 60 s, annealing at 60 °C for 60 s, and extension at 72 °C for 60 s; and (3) Extension at 72 °C for 10 min. PCR products were confirmed by agarose gel electrophoresis. Ten percent of the samples were randomly selected for repeated genotyping and the results were 100 percent consistent.

Statistical analysis

The socio-demographic data and clinical data between case and control subjects were compared by the chi-square test. A goodness-of-fit chi-squared test was also used to analyze whether the SNP (XPG rs2094258, rs751402, rs2296147, rs1047768 and rs873601) distributions conform to the Hardy-Weinberg equilibrium (HWE) in controls. Odds ratios (ORs) and 95% confidence intervals (CIs) for examined SNPs were analyzed by Logistic regression method and adjusted by age, gender, and *H. pylori* infection status. The heterozygous model, homozygous model, recessive model, and dominant model were all analyzed for five SNPs. For rs2094258, heterozygous model, homozygous model, recessive model, and dominant model were CT *vs* CC, TT *vs* CC, TT *vs* CT + CC, TT + CT *vs* CC, respectively. Kaplan-Meier method and log-rank test were used for plotting cases' survival curves and for comparisons, respectively.

Multivariate Cox regression was used for exploring possible prognostic factors, which included gender, age, drinking, smoking, *H. pylori*, TNM stage, tumor differentiation, lymphovascular invasion, neural invasion, and chemotherapy. SPSS 24.0 statistical software was used for all statistical analyses (Statistical Package for the Social Sciences, version 24, SSPS Inc, Chicago, IL, United States). All statistical tests were two-sided, with $P < 0.05$ as the boundary value.

RESULTS

The majority of study participants were male and less than age 60, with no significant differences in these factors between cases and controls (Table 1). All five loci in the control group were consistent with the Hardy-Weinberg equilibrium. The rate of *H. pylori* infection in GC patients was significantly higher than that in the control group (70.6% *vs* 53.6%, $P < 0.001$). None of the associations between studied loci (rs2094258, rs751402, rs2296147, rs1047768, and rs873601) and GC risk was statistically significant (Table 2). However, the rs2296147 CC genotype frequency was higher in the GC case

group than that in the control group (5.9% *vs* 3.9%, $P = 0.050$). After adjustment for age, sex, and *H. pylori* status, this genotype was marginally associated with a slightly higher risk of GC (OR = 1.40, 95%CI: 0.97-2.50, $P = 0.061$) than the TT genotype. The CC genotype was found to be marginally associated with a higher risk of GC (OR = 1.36, 95%CI = 0.99-2.49, $P = 0.053$) in the recessive model (CC *vs* CT + TT).

As the number of C alleles of rs2094258 increased, the survival rate of GC cases decreased (log-rank test, $P = 0.032$) (Table 3). Among all the cases, mortality was 45.4% with rs2094258 TT genotype (no C allele), 53.7% with CT genotype (one C allele), and 59.4% with CC genotype (two C alleles). The other four SNP loci (rs751402, Rs2296147, rs1047768, and rs873601) had no significant correlation with overall survival in patients with GC. For rs2094258, survival curves varied significantly with genotype (log-rank test, $P = 0.032$) (Figure 1A). GC patients with the rs2094258 CT + CC genotype showed a lower survival than patients with the TT genotype (log-rank test, $P = 0.028$) (Figure 1B). Cases with the CC genotype had a poorer prognosis than those with the TT + CT genotype (log-rank test, $P = 0.039$) (Figure 1C).

In univariate survival analysis, age ($P = 0.011$), TNM stage ($P < 0.001$), no chemotherapy ($P < 0.001$), poor differentiation ($P < 0.001$), neural invasion ($P < 0.001$), and lymphovascular invasion ($P < 0.001$) were positively associated with the 5-year survival of GC cases (Table 4). Gender and *H. pylori* status were not significantly associated with the 5-year survival of GC cases. In multivariate analysis, the increase in the number of C alleles of rs2094258 (hazard ratio [HR] = 1.19, 95%CI: 1.02-1.45, $P = 0.037$), chemotherapy (HR = 0.81, 95%CI: 0.63-0.98; $P = 0.041$), differentiation (HR = 4.51, 95%CI: 1.99-8.23, $P < 0.001$), and lymphovascular invasion (HR = 1.97, 95%CI: 1.44-3.01, $P < 0.001$) were associated with survival in GC cases (Table 5).

DISCUSSION

We genotyped five functional SNPs in the *XPG* gene involved in the NER pathway and assessed their associations with GC risk and survival in China. Although rs2296147 was marginally associated with risk of GC ($P = 0.05$), we found no statistically significant associations. However, as the number of C alleles of rs2094258 increased, the survival of GC cases showed a decreasing trend. Poor differentiation, lymphovascular invasion, no chemotherapy, and increase in the number of C alleles of rs2094258 were associated with decreased survival among cases.

Few studies have explored the association of *XPG* SNPs with survival in GC patients. Our results are similar to those of Liu *et al*[25], who analysed the association between *XPG* SNPs and survival among 373 GC patients in China. That study found that in univariate model, the survival rate of those with the *XPG* rs2094258 AG genotype was higher than that of wild-type GG carriers (HR = 0.59, 95%CI: 0.39-0.90, $P = 0.014$), and that in multivariate analysis, the AA + AG genotype presented a significant survival advantage over the GG genotype (adjusted HR = 0.65, 95%CI: 0.44-0.97)[25]. Liu *et al*[25] also found that the AA + AG genotype of the *XPG* rs2094258 polymorphism improved survival in those with the following characteristics: Age more than 60, lymphatic metastasis, TNM stage III-IV, Borrmann III–IV, and diffuse-type gastric tumors[25].

Consistent with our study, a recent meta-analysis did not observe an overall association between the *XPG* rs2094258 SNPs and GC risk[26]. Some studies have found associations between the rs2094258 polymorphism and GC risk[22,26-28]. For example, Yang *et al*[23] assessed three *XPG* SNPs (rs2296147T>C, rs2094258C>T, and rs873601G>A) and found that the rs2094258 C>T polymorphism was associated with an increased GC risk. Meanwhile, *H. pylori*-infected individuals with the rs2094258 TT genotype had a much higher GC risk (OR = 2.13, 95%CI: 1.22-3.35, P for interaction = 0.030)[23]. However, *H. pylori* status did not modify associations with this SNP in our data. Varying cofactors among study populations, small sample size, the use of different PCR methods, or the low penetrance of this SNP may account for the inconsistent findings[15].

XPG rs2296147 was not associated with GC in the present study, a finding consistent with those of other studies[24,25]. However, significant associations between rs2296147 polymorphisms and GC risk were observed in Asian populations in numerous studies in one meta-analysis (CT *vs* TT: OR = 0.93, 95%CI: 0.87-0.99, $P = 0.036$)[29]. Further, the rs2296147 CC genotype was associated with a reduced risk of GC in a Chinese population (OR = 0.52, 95%CI: 0.27-0.97)[23]. These differences may be due to regional differences, small sample size, or heterogeneity of clinical feature[26].

The NER pathway belongs to the DNA repair pathways, and its functions include removing exogenous or endogenous DNA damage or adduct between chains and recruiting proliferating-cell nuclear antigen to the damage site for the subsequent gap-

Table 1 Demographic characteristics of the participants

		Case	Control	X^2	*P*-value
		n = 956	*n* = 1012		
Age (yr)					
	≤60	569 (59.5)	637 (62.9)	2.289	0.130
	>60	387 (40.5)	375 (37.1)		
Gender					
	Male	667 (69.8)	724 (71.5)	0.662	0.416
	Female	289 (30.2)	288 (28.5)		
H. pylori infection					
	Positive	675 (70.6)	542 (53.6)	59.835	<0.001
	Negative	281 (29.4)	470 (46.4)		
Drinking					
	No	758 (79.3)	497 (49.1)	2.787	0.095
	Yes	198 (20.7)	515 (50.9)		
Smoking					
	No	560 (58.6)	517 (51.1)	1.382	0.240
	Yes	396 (41.4)	495 (48.9)		

H. pylori: *Helicobacter pylori*.

filling DNA synthesis[30]. DNA repair usually includes two stages of excision and repair synthesis[31]. The *XPG* gene locating at chromosome 13q33 consists of 15 exons[10], which is considered to cut the DNA at the 3′ terminus, initiate transcription-coupled DNA repair, and participate in RNA polymerase II transcription[32]. In the process of DNA repair, XPG binds to XPB as part of the transcription factor IIH (TFIIH) complex and strongly interacts with the TFIIH complex, a multi-subunit complex located at the intersection of transcription and DNA repair[33], which is involved in the DNA demethylation induced by overexpression of Gadd45a[10]. Meanwhile, XPG-related nucleases are used hierarchically for the excision of double-stranded rDNA break resection[34]. Recent report suggests that XPG incises the R-loop structure and participates in the RAD52-dependent resolution of DNA-RNA hybrids[35]. The rs1047768, rs751402, and rs2296147 are located in the exon 2, proximal promoter, and 5' untranslated region of the gene, respectively[36,37]. Based on the dbSNP database, the rs2094258 located at the *XPG* gene intron region participates in the initiation of the transcription-coupled DNA repair.

The limitations of this study are as follows. First, we could not explore and determine the exact mechanism by which *XPG* SNPs influence GC survival. Second, this study only examined five functional SNPs and did not include all the SNPs in the *XPG* gene that might play a key role in GC development. Third, selection bias and information bias are inherent threats to case-control studies, and cannot be ruled out in our study. Genetic factors have been shown to play key roles in the development, progression, and prognosis of GC. If confirmed by other studies, the results of our study suggest that *XPG* rs2094258 polymorphisms may serve as genetic biomarkers for GC prognosis, and may provide clues to biological underpinnings of GC progression.

Table 2 Single nucleotide polymorphism and genotype distribution between gastric cancer cases and controls

SNP	Genotype	Case	Control	HWE	*P*-value	OR	95%CI	*P*-value	OR[1]	95%CI	*P*-value
		n = 956	*n* = 1012								
Rs2094258	CC	390 (40.8)	437 (43.2)	0.318	0.853	1.00					
	CT	441 (46.1)	464 (45.8)			1.07	0.88-1.29	0.545	1.05	0.86-1.31	0.626
	TT	125 (13.1)	111 (11.0)			1.26	0.94-1.69	0.133	1.19	0.90-1.20	0.249
	Dominant	566 (59.2)	575 (56.8)			1.10	0.92-1.32	0.284	1.06	0.87-1.37	0.388
	Recessive	831 (86.9)	901 (89.0)			1.22	0.93-1.60	0.150	1.19	0.91-1.72	0.303
Rs751402	CC	366 (38.3)	371 (36.7)	0.183	0.913	1.00					
	CT	467 (48.8)	476 (47.0)			0.95	0.78-1.16	0.618	0.98	0.77-1.33	0.774
	TT	123 (12.9)	165 (16.3)			0.88	0.67-1.15	0.341	0.90	0.61-1.28	0.491
	Dominant	590 (61.7)	632 (62.5)			0.95	0.79-1.14	0.554	0.95	0.78-1.22	0.711
	Recessive	833 (87.1)	847 (83.7)			0.90	0.71-1.15	0.411	0.93	0.69-1.18	0.487
Rs2296147	TT	599 (62.7)	640 (63.2)	0.076	0.963	1.00					
	CT	301 (31.5)	332 (32.9)			0.97	0.80-1.17	0.745	0.97	0.80-1.19	0.674
	CC	56 (5.9)	40 (3.9)			1.50	0.98-2.28	0.059	1.40	0.97-2.50	0.061
	Dominant	357 (37.3)	372 (36.8)			1.03	0.85-1.23	0.789	1.01	0.80-1.38	0.865
	Recessive	900 (94.1)	973 (96.1)			1.51	1.00-2.29	0.050	1.36	0.99-2.49	0.053
Rs1047768	TT	505 (52.8)	540 (53.4)	0.367	0.832	1.00					
	CT	379 (39.6)	406 (40.1)			1.00	0.83-1.20	0.985	1.00	0.80-1.28	0.989
	CC	72 (7.6)	66 (6.5)			1.17	0.82-1.66	0.395	1.11	0.79-1.92	0.505
	Dominant	451 (47.2)	472 (46.6)			1.02	0.86-1.22	0.812	1.01	0.81-1.45	0.897
	Recessive	884 (92.5)	946 (93.5)			1.17	0.83-1.65	0.381	1.12	0.74-1.84	0.561
Rs873601	GG	271 (28.3)	288 (28.5)	0.247	0.884	1.00					
	AG	475 (49.7)	514 (50.8)			0.98	0.80-1.21	0.865	0.99	0.63-1.88	0.931
	AA	210 (22.0)	210 (20.7)			1.06	0.83-1.37	0.638	1.03	0.71-2.01	0.807
	Dominant	685 (71.7)	724 (71.5)			1.12	0.92-1.37	0.245	1.07	0.82-1.94	0.466
	Recessive	746 (78.0)	802 (79.2)			1.08	0.87-1.33	0.511	0.98	0.88-1.37	0.685

[1]OR was calculated by Logistic regression method after adjusting for age and gender. *H. pylori*: *Helicobacter pylori* infection status; SNP: Single nucleotide polymorphism; HWE: Hardy-Weinberg equilibrium; OR: Odds ratio.

Table 3 Genotypes of single nucleotide polymorphism and 5-year survival status of gastric cancer cases

SNP	Genotype	Case	Death (%)	5-yr survival rate (%)	*P*-value[1]
Rs2094258	CC	388	157 (40.5)	59.4	0.032
	CT	440	203 (46.1)	53.7	
	TT	123	68 (55.3)	45.4	
Rs751402	CC	363	162 (44.6)	55.3	0.523
	CT	465	216 (46.5)	53.3	
	TT	121	50 (41.3)	58.9	
Rs2296147	TT	589	281 (47.7)	52.0	0.217
	CT	297	124 (41.8)	57.6	
	CC	50	23 (46.0)	57.7	
Rs1047768	TT	501	243 (48.5)	51.2	0.102
	CT	375	152 (40.5)	59.1	
	CC	67	33 (49.3)	53.4	
Rs873601	GG	268	131 (48.9)	51.2	0.362
	AG	472	203 (43.0)	56.8	
	AA	207	94 (45.4)	54.7	

[1]*P* was calculated by log-rank test. SNP: Single nucleotide polymorphism.

Table 4 Univariate analysis of associations between influencing factors and 5-year survival of gastric cancer cases

Variable	Classification	*n*	Death (%)	5-yr survival rate (%)	*P*-value[1]
Gender	Male	667	303 (45.4)	54.2	0.399
	Female	289	125 (43.3)	56.1	
Age (yr)	≤60	569	235 (41.3)	58.5	0.011
	>60	387	193 (49.9)	49.7	
Drinking	No	758	335 (44.2)	55.6	0.259
	Yes	198	93 (47.0)	52.9	
Smoking	No	560	244 (43.6)	55.9	0.417
	Yes	396	184 (46.5)	53.3	
H. pylori infection	Negative	281	118 (42.0)	58.1	0.284
	Positive	675	310 (45.9)	51.4	
TNM stage	I	178	18 (10.1)	87.7	<0.001
	II	366	129 (35.2)	66.9	
	III	404	281 (69.6)	29.8	
Differentiation	Poor	661	329 (49.8)	50.0	<0.001
	Moderate or high	280	99 (35.4)	66.7	
Lymphovascular invasion	Negative	269	57 (21.2)	79.6	<0.001
	Positive	675	371 (55.0)	44.8	
Neural invasion	Negative	396	132 (33.3)	67.4	<0.001
	Positive	548	296 (54.0)	45.7	
Chemotherapy	No	361	281 (77.8)	22.9	<0.001
	Yes	592	147 (24.8)	75.1	

Missing values: 8 for tumor–node–metastasis stage, 15 for differentiation, 12 for lymphovascular invasion and neural invasion, and 3 for chemotherapy.
[1]*P* was calculated by log-rank test. *H. pylori*: *Helicobacter pylori*; TNM: Tumor–node–metastasis.

Table 5 Multivariate analysis of gastric cancer survival

Variable	Comparison	HR[1]	95%CI	*P*-value
Differentiation	Poor *vs* Moderate or high	4.51	1.99-8.23	<0.001
Lymphovascular invasion	Positive *vs* Negative	1.97	1.44-3.01	<0.001
Chemotherapy	Yes *vs* No	0.81	0.63-0.98	0.041
Rs2094258	Increase in C allele	1.19	1.02-1.45	0.037

[1]HR was calculated by Cox regression model. HR: Hazard ratio.

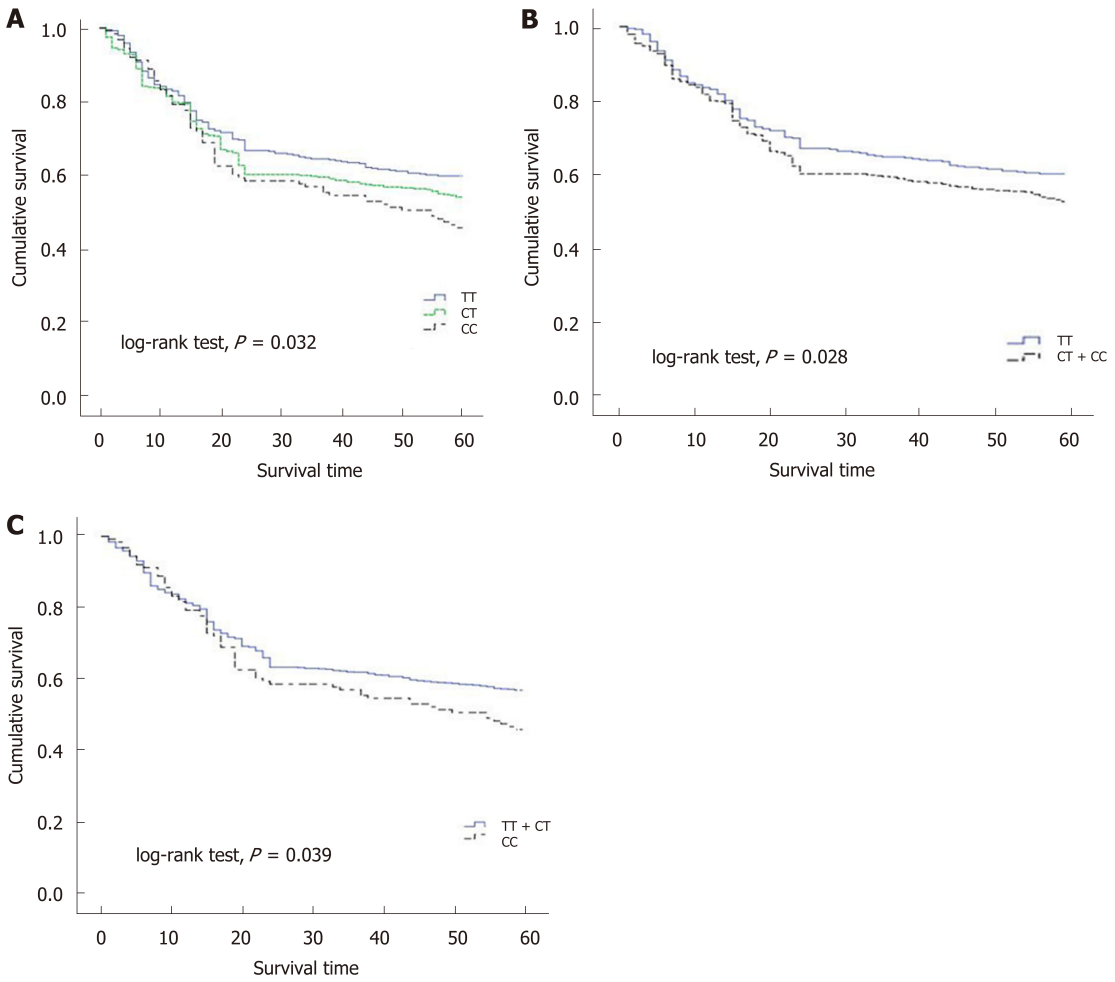

Figure 1 Survival plots of gastric cancer cases stratified by rs2094258 genotype. A: Compared with the survival of cases with no C allele (genotype TT, blue line), cases with one C allele (genotype CT, green line) demonstrated a decreased survival rate and cases with two C alleles (genotype CC, dotted line) exhibited the lowest survival rate; B: Compared with the survival of cases with no C allele (genotype TT, blue line), cases with recessive allele (genotype CT + CC, dotted line) demonstrated a decreased survival rate; C: In the dominant model, case with CC allele showed a lower survival rate compared with cases with dominant allele.

ARTICLE HIGHLIGHTS

Research background

Gastric cancer (GC) is one of the leading causes of cancer-related death worldwide, which causes a high social and economic burden. Xeroderma pigmentosum group G (XPG) or ERCC5 may play a key role in DNA repair, thus affecting cancer prognosis. Studies have shown that *XPG* SNPs may affect the development of cancer such as lung cancer, colorectal cancer, and breast cancer.

Research motivation

Only a few studies have explored the relationships between *XPG* gene SNPs and GC, and the results are inconsistent.

Research objectives

To examine GC risk and survival in relation to common functional *XPG* SNPs through a case-control study, which might improve our understanding of GC and provide new therapeutic targets for this malignancy.

Research methods

A total of 956 histologically confirmed GC cases and 1012 controls were matched by sex, age (within 5 years), and residential district. Cases were followed and the survival time was recorded. DNA was extracted from peripheral blood samples of all cases and controls. *XPG* rs2094258, rs751402, rs2296147, rs1047768, and rs873601 polymorphisms were genotyped using PCR-RFLP. Logistic regression and Cox regression were used for analyzing associations of *XPG* SNPs with risk of GC and prognosis, respectively.

Research results

We found that GC patients with the rs2094258 CT + CC genotype showed a worse survival than those with the TT genotype, and patients with the CC genotype had a tendency of unfavorable prognosis compared with those with the TT + CT genotype. The increase in the number of C alleles of rs2094258 was associated with the long-term survival of GC cases. Other risk factors for survival included tumor differentiation, lymphovascular invasion, and use of chemotherapy. However, the exact mechanisms by which *XPG* SNPs influence GC survival remain to be solved.

Research conclusions

The *XPG* rs2094258 polymorphism may be associated with overall survival in patients with GC.

Research perspectives

If confirmed by other studies, the results of our study suggest that *XPG* rs2094258 polymorphisms may serve as genetic biomarkers for GC prognosis, and may provide clues to biological underpinnings of GC progression.

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REFERENCES

1 **Van Cutsem E**, Sagaert X, Topal B, Haustermans K, Prenen H. Gastric cancer. *Lancet* 2016; **388**: 2654-2664 [PMID: 27156933 DOI: 10.1016/S0140-6736(16)30354-3]
2 **World Health Organization**. Cancer [cited 12 September 2018]. Available from: http://www.who.int/mediacentre/factsheets/fs297/en/
3 **Ang TL**, Fock KM. Clinical epidemiology of gastric cancer. *Singapore Med J* 2014; **55**: 621-628 [PMID: 25630323 DOI: 10.11622/smedj.2014174]
4 **Ferlay J**, Soerjomataram I, Dikshit R, Eser S, Mathers C, Rebelo M, Parkin DM, Forman D, Bray F. Cancer incidence and mortality worldwide: Sources, methods and major patterns in GLOBOCAN 2012. *Int J Cancer* 2015; **136**: E359-E386 [PMID: 25220842 DOI: 10.1002/ijc.29210]
5 **Liang J**, Xu YY, Zhang C, Xia QR. Association of XPG gene rs751402 polymorphism with gastric cancer risk: A meta-analysis in the Chinese population. *Int J Biol Markers* 2018; **33**: 174-179 [PMID: 29148016 DOI: 10.5301/ijbm.5000313]
6 **Casamayor M**, Morlock R, Maeda H, Ajani J. Targeted literature review of the global burden of gastric cancer. *Ecancermedicalscience* 2018; **12**: 883 [PMID: 30679950 DOI: 10.3332/ecancer.2018.883]
7 **Ferro A**, Peleteiro B, Malvezzi M, Bosetti C, Bertuccio P, Levi F, Negri E, La Vecchia C, Lunet N. Worldwide trends in gastric cancer mortality (1980-2011), with predictions to 2015, and incidence by subtype. *Eur J Cancer* 2014; **50**: 1330-1344 [PMID: 24650579 DOI: 10.1016/j.ejca.2014.01.029]
8 **Qian X**, Tan H, Zhang J, Liu K, Yang T, Wang M, Debinskie W, Zhao W, Chan MD, Zhou X. Identification of biomarkers for pseudo and true progression of GBM based on radiogenomics study. *Oncotarget* 2016; **7**: 55377-55394 [PMID: 27421136 DOI: 10.18632/oncotarget.10553]
9 **Apostolou Z**, Chatzinikolaou G, Stratigi K, Garinis GA. Nucleotide Excision Repair and Transcription-Associated Genome Instability. *Bioessays* 2019; **41**: e1800201 [PMID: 30919497 DOI: 10.1002/bies.201800201]
10 **Sugasawa K**. Xeroderma pigmentosum genes: Functions inside and outside DNA repair. *Carcinogenesis* 2008; **29**: 455-465 [PMID: 18174245 DOI: 10.1093/carcin/bgm282]
11 **Wakasugi M**, Reardon JT, Sancar A. The non-catalytic function of XPG protein during dual incision in human nucleotide excision repair. *J Biol Chem* 1997; **272**: 16030-16034 [PMID: 9188507 DOI: 10.1074/jbc.272.25.16030]
12 **Duan WX**, Hua RX, Yi W, Shen LJ, Jin ZX, Zhao YH, Yi DH, Chen WS, Yu SQ. The association between OGG1 Ser326Cys polymorphism and lung cancer susceptibility: A meta-analysis of 27 studies. *PLoS One* 2012; **7**: e35970 [PMID: 22540013 DOI: 10.1371/journal.pone.0035970]
13 **Lawania S**, Singh N, Behera D, Sharma S. XPG polymorphisms and their association with lung cancer susceptibility, overall survival and response in North Indian patients treated with platinum-based doublet chemotherapy. *Future Oncol* 2019; **15**: 151-165 [PMID: 30522358 DOI: 10.2217/fon-2018-0408]
14 **Wang F**, Zhang SD, Xu HM, Zhu JH, Hua RX, Xue WQ, Li XZ, Wang TM, He J, Jia WH. XPG rs2296147 T>C polymorphism predicted clinical outcome in colorectal cancer. *Oncotarget* 2016; **7**: 11724-11732 [PMID: 26887052 DOI: 10.18632/oncotarget.7352]
15 **Su J**, Zhu Y, Dai B, Yuan W, Song J. XPG Asp1104His polymorphism increases colorectal cancer risk especially in Asians. *Am J Transl Res* 2019; **11**: 1020-1029 [PMID: 30899401]
16 **Wang H**, Wang T, Guo H, Zhu G, Yang S, Hu Q, Du Y, Bai X, Chen X, Su H. Association analysis of ERCC5 gene polymorphisms with risk of breast cancer in Han women of northwest China. *Breast Cancer* 2016; **23**: 479-485 [PMID: 25644244 DOI: 10.1007/s12282-015-0590-2]
17 **He J**, Wang F, Zhu J, Zhang R, Yang T, Zou Y, Xia H. Association of potentially functional variants in the XPG gene with neuroblastoma risk in a Chinese population. *J Cell Mol Med* 2016; **20**: 1481-1490 [PMID: 27019310 DOI: 10.1111/jcmm.12836]
18 **Al Sayed Ahmed H**, Raslan WF, Deifalla AHS, Fathallah MD. Overall survival of classical Hodgkins lymphoma in Saudi patients is affected by XPG repair gene polymorphism. *Biomed Rep* 2019; **10**: 10-16 [PMID: 30588297 DOI: 10.3892/br.2018.1165]
19 **Zavras AI**, Yoon AJ, Chen MK, Lin CW, Yang SF. Association between polymorphisms of DNA repair gene ERCC5 and oral squamous cell carcinoma. *Oral Surg Oral Med Oral Pathol Oral Radiol* 2012; **114**: 624-629 [PMID: 22981091 DOI: 10.1016/j.oooo.2012.05.013]
20 **Yang LQ**, Zhang Y, Sun HF. Investigation on ERCC5 genetic polymorphisms and the development of gastric cancer in a Chinese population. *Genet Mol Res* 2016; 15 [PMID: 27706622 DOI: 10.4238/gmr.15038364]

21 **Lu JJ**, Zhang HQ, Mai P, Ma X, Chen X, Yang YX, Zhang LP. Lack of association between ERCC5 gene polymorphisms and gastric cancer risk in a Chinese population. *Genet Mol Res* 2016; 15 [PMID: 27323158 DOI: 10.4238/gmr.15027779]

22 **Feng YB**, Fan DQ, Yu J, Bie YK. Association between XPG gene polymorphisms and development of gastric cancer risk in a Chinese population. *Genet Mol Res* 2016; 15 [PMID: 27323165 DOI: 10.4238/gmr.15027877]

23 **Yang WG**, Zhang SF, Chen JW, Li L, Wang WP, Zhang XF. SNPs of excision repair cross complementing group 5 and gastric cancer risk in Chinese populations. *Asian Pac J Cancer Prev* 2012; **13**: 6269-6272 [PMID: 23464443 DOI: 10.7314/APJCP.2012.13.12.6269]

24 **Chen YZ**, Guo F, Sun HW, Kong HR, Dai SJ, Huang SH, Zhu WW, Yang WJ, Zhou MT. Association between XPG polymorphisms and stomach cancer susceptibility in a Chinese population. *J Cell Mol Med* 2016; **20**: 903-908 [PMID: 26820236 DOI: 10.1111/jcmm.12773]

25 **Liu J**, Deng N, Xu Q, Sun L, Tu H, Wang Z, Xing C, Yuan Y. Polymorphisms of multiple genes involved in NER pathway predict prognosis of gastric cancer. *Oncotarget* 2016; **7**: 48130-48142 [PMID: 27340861 DOI: 10.18632/oncotarget.10173]

26 **Zhang Z**, Yin J, Xu Q, Shi J. Association between the XPG gene rs2094258 polymorphism and risk of gastric cancer. *J Clin Lab Anal* 2018; **32**: e22564 [PMID: 29732643 DOI: 10.1002/jcla.22564]

27 **He J**, Qiu LX, Wang MY, Hua RX, Zhang RX, Yu HP, Wang YN, Sun MH, Zhou XY, Yang YJ, Wang JC, Jin L, Wei QY, Li J. Polymorphisms in the XPG gene and risk of gastric cancer in Chinese populations. *Hum Genet* 2012; **131**: 1235-1244 [PMID: 22371296 DOI: 10.1007/s00439-012-1152-8]

28 **Hua RX**, Zhuo ZJ, Zhu J, Jiang DH, Xue WQ, Zhang SD, Zhang JB, Li XZ, Zhang PF, Jia WH, Shen GP, He J. Association between genetic variants in the XPG gene and gastric cancer risk in a Southern Chinese population. *Aging (Albany NY)* 2016; **8**: 3311-3320 [PMID: 27929383 DOI: 10.18632/aging.101119]

29 **Han C**, Huang X, Hua R, Song S, Lyu L, Ta N, Zhu J, Zhang P. The association between XPG polymorphisms and cancer susceptibility: Evidence from observational studies. *Medicine (Baltimore)* 2017; **96**: e7467 [PMID: 28796034 DOI: 10.1097/MD.0000000000007467]

30 **Schärer OD**. XPG: Its products and biological roles. *Adv Exp Med Biol* 2008; **637**: 83-92 [PMID: 19181113 DOI: 10.1007/978-0-387-09599-8_9]

31 **Michalska MM**, Samulak D, Romanowicz H, Jabłoński F, Smolarz B. Association between single nucleotide polymorphisms (SNPs) of XRCC2 and XRCC3 homologous recombination repair genes and ovarian cancer in Polish women. *Exp Mol Pathol* 2016; **100**: 243-247 [PMID: 26801223 DOI: 10.1016/j.yexmp.2016.01.007]

32 **Clarkson SG**. The XPG story. *Biochimie* 2003; **85**: 1113-1121 [PMID: 14726017 DOI: 10.1016/j.biochi.2003.10.014]

33 **Kolesnikova O**, Radu L, Poterszman A. TFIIH: A multi-subunit complex at the cross-roads of transcription and DNA repair. *Adv Protein Chem Struct Biol* 2019; **115**: 21-67 [PMID: 30798933 DOI: 10.1016/bs.apcsb.2019.01.003]

34 **Barnum KJ**, Nguyen YT, O'Connell MJ. XPG-related nucleases are hierarchically recruited for double-stranded rDNA break resection. *J Biol Chem* 2019; **294**: 7632-7643 [PMID: 30885940 DOI: 10.1074/jbc.RA118.005415]

35 **Kato R**, Miyagawa K, Yasuhara T. The role of R-loops in transcription-associated DNA double-strand break repair. *Mol Cell Oncol* 2019; **6**: 1542244 [PMID: 30788418 DOI: 10.1080/23723556.2018.1542244]

36 **Yoon AJ**, Kuo WH, Lin CW, Yang SF. Role of ERCC5 polymorphism in risk of hepatocellular carcinoma. *Oncol Lett* 2011; **2**: 911-914 [PMID: 22866149 DOI: 10.3892/ol.2011.325]

37 **Blomquist TM**, Crawford EL, Willey JC. Cis-acting genetic variation at an E2F1/YY1 response site and putative p53 site is associated with altered allele-specific expression of ERCC5 (XPG) transcript in normal human bronchial epithelium. *Carcinogenesis* 2010; **31**: 1242-1250 [PMID: 20233728 DOI: 10.1093/carcin/bgq057]

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ORIGINAL ARTICLE

Retrospective Study

Timing, distribution, and microbiology of infectious complications after necrotizing pancreatitis

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Abstract

BACKGROUND

Acute pancreatitis (AP) is a common acute abdominal disease worldwide, and its incidence rate has increased annually. Approximately 20% of AP patients develop into necrotizing pancreatitis (NP), and 40% to 70% of NP patients have infectious complications, which usually indicate a worse prognosis. Infection is an important sign of complications in NP patients.

AIM

To investigate the difference in infection time, infection site, and infectious strain in NP patients with infectious complications.

METHODS

The clinical data of AP patients visiting the Department of General Surgery of Xuanwu Hospital of Capital Medical University from January 1, 2014 to December 31, 2018 were collected retrospectively. Enhanced computerized tomography or magnetic resonance imaging findings in patients with NP were included in the study. Statistical analysis of infectious bacteria, infection site, and infection time in NP patients with infectious complications was performed, because knowledge about pathogens and their antibiotic susceptibility patterns is essential for selecting an appropriate antibiotic. In addition, the factors that might influence the prognosis of patients were analyzed.

RESULTS

In this study, 539 strains of pathogenic bacteria were isolated from 162 patients with NP infection, including 212 strains from pancreatic infections and 327 strains from extrapancreatic infections. Gram-negative bacteria were the main infectious species, the most common of which were *Escherichia coli* and

Data sharing statement: The original anonymous dataset is available on request from the corresponding author at feili36@ccmu.edu.cn.

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Pseudomonas aeruginosa. The extrapancreatic infection time (9.1 ± 8.8 d) was earlier than the pancreatic infection time (13.9 ± 12.3 d). Among NP patients with early extrapancreatic infection (< 14 d), bacteremia (25.12%) and respiratory tract infection (21.26%) were predominant. Among NP patients with late extrapancreatic infection (> 14 d), bacteremia (15.94%), respiratory tract infection (7.74%), and urinary tract infection (7.71%) were predominant. Drug sensitivity analysis showed that *P. aeruginosa* was sensitive to enzymatic penicillins, third- and fourth-generation cephalosporins, and carbapenems. *Acinetobacter baumannii* and *Klebsiella pneumoniae* were sensitive only to tigecycline; *Staphylococcus epidermidis* and *Enterococcus faecium* were highly sensitive to linezolid, tigecycline, and vancomycin.

CONCLUSION
In this study, we identified the timing, the common species, and site of infection in patients with NP.

Key words: Necrotizing pancreatitis; Extrapancreatic infection; Pathogenic bacteria; Drug sensitivity test

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Core tip: In our study, Gram-negative bacteria were the main pathogens in necrotizing pancreatitis patients with infectious complications in our hospital. The most common Gram-negative bacteria were *Escherichia coli* and *Pseudomonas aeruginosa*. Additionally, the proportion of multidrug-resistant bacteria was relatively large, and caution should be used in the application of antibiotics. The extrapancreatic infection time was usually earlier than that of pancreatic infection. For patients with suspected infection, blood and respiratory pathogens should be cultured first. Third- or fourth-generation cephalosporins or carbapenems can be used as empirical drugs. Persistent organ failure, multidrug resistance, and multiple operations were risk factors for death.

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INTRODUCTION

Acute pancreatitis (AP) is a common acute abdominal disease worldwide, and its incidence rate has increased annually[1]. With the progression of the disease, 20% of AP patients develop into necrotizing pancreatitis (NP). Approximately 40% to 70% of NP patients have infectious complications, which usually indicate a worse prognosis. Infection is an important sign of complications in NP patients[1-5]. To date, studies on pancreatic infection have mainly focused on pancreatic and peripancreatic infection, but few have focused on extrapancreatic infection and coinfection.

Therefore, this study collected clinical data of NP patients who visited our hospital from January 1, 2014 to December 31, 2018. The aim was to analyze the difference in infection time, infection site, and bacterial strains in NP patients with infectious complications and the influence of these factors on mortality, in order to help determine treatment strategies for NP patients with coinfection.

MATERIALSANDMETHODS

Patients

The clinical data of NP patients who visited our hospital from January 1, 2014 to December 31, 2018 were retrospectively collected.

Inclusion criteria

Patients diagnosed with NP by enhanced computerized tomography (CT) or magnetic resonance imaging (MRI) were included in the study.

Exclusion criteria

The exclusion criteria were: (1) Acute attack of chronic pancreatitis; (2) Abdominal exploratory surgery due to pancreatitis; (3) Pancreatitis due to abdominal surgery; and (4) Acute abdominal events such as visceral organ perforation, bleeding, and abdominal compartment syndrome

Definitions

AP: AP can be diagnosed by any two of the following three items: (1) Acute epigastric pain, radiating to the waist and back; (2) Blood amylase and/or lipase levels at least three times higher than the normal limit; and (3) The presence of typical pancreatic lesions upon imaging examination (such as abdominal ultrasound, enhanced CT, or MRI).

NP: NP presents inflammation associated with pancreatic parenchymal necrosis and/or peripancreatic necrosis. On contrast-enhanced CT, there is lack of pancreatic parenchymal enhancement by intravenous contrast agent and/or the presence of findings of peripancreatic necrosis[3]. According to Balthazar *et al*[6], the degree of pancreatic necrosis in NP patients is classified as < 30%, 30%-50%, and > 50%.

Infectious NP (INP): In clinical treatment, pancreatic infection is suspected in NP patients when one of the following symptoms is present: (1) Sudden high fever (> 38.5 °C) or persistent fever (> 38.5 °C) that does not return; (2) A significant increase in leukocyte count, or the percentage of neutrophils and inflammatory markers (CRP, IL-6, and PCT); and (3) Continuous deterioration of clinical symptoms such as new-onset organ failure or multiple organ failure. Diagnostic methods include the following: (1) Enhanced CT showing gas configurations in the necrotic collection; and (2) Positive culture from pancreatic necrosis obtained by puncture or operation.

Extrapancreatic infection: Extrapancreatic infection is defined as infection occurring in other organs of AP patients. Common sites of extrapancreatic infection include the blood, respiratory tract, urinary tract, abdominal cavity, biliary tract, and surgical incision site[7-9]. When infection symptoms are suspected in AP patients, but no definite signs of infection (bubbles) are found in the necrotic pancreatic tissue on imaging, and the culture of necrotic tissue and pus from puncture and drainage is negative, suspected patients with extrapancreatic infection can be diagnosed or excluded by multiple, multisite pathogen culture[4,5,7-9].

Multidrug-resistant (MDR) bacteria: MDR bacteria, which are bacteria that are usually sensitive to three or more commonly used antibiotics but show simultaneous resistance to these drugs, were evaluated according to the Chinese expert consensus on the prevention and control of nosocomial infection by MDR bacteria.

Management of patients

After admission, patients with confirmed NP were treated conservatively with analgesics, fluid resuscitation, and supportive treatment. Patients with clinically severe AP (suspected or confirmed symptoms of organ failure) were admitted to the intensive care unit (ICU) with all possible organ support systems, including ventilatory support, vasopressors, and dialysis as and when required. Patients with sterile necrosis are usually treated conservatively. For NP patients with suspected or confirmed infectious complications, broad-spectrum antibiotics (such as third- and fourth-generation cephalosporins and carbapenems) were used to control infection, and sensitive antibiotics were selected according to the drug sensitivity results of the patients. The indications for surgical intervention in NP patients were as follows: (1) After conservative treatment, the clinical symptoms did not improve significantly or worsened continuously; (2) Pancreatic infection was confirmed in the patient; and (3) The range of sterile necrosis in the patient was enlarged, and the surrounding organs were compressed. Minimally invasive approaches to remove necrotic tissue are usually selected according to the necrotic site in the patient.

Statistical analysis

Data were input into Excel 2018, the baseline data are recorded as the mean ± SD or medians (ranges) as appropriate. Independent samples *t*-tests were performed to analyze the differences between the groups. Qualitative data were scored, and chi-square or Fisher's exact tests were performed to analyze the differences between the groups. Logistic regression was used to determine the risk of death in the patients *via* SPSS 23.0 statistical software, with the level of statistical significance set as $P < 0.05$.

RESULTS

We collected the clinical data of patients with AP who visited our department from January 1, 2014 to December 31, 2018. A total of 205 NP patients were enrolled according to the results of enhanced CT in our hospital or another hospital. There were 140 males and 65 females, with an average age of 49.5 ± 15.7 years. The causes of NP were gallstones in 109 patients, hyperlipidemia in 67 patients, unknown etiology in 12 patients, alcoholism in 10 patients, endoscopic retrograde cholangiopancreatography in 6 patients, and posttraumatic pancreatitis in 1 patient. There was no significant difference in age, sex, or etiology between the infection group and the non-infection group (Table 1).

Among the 205 NP patients, 163 had infectious complications, and 42 had sterile necrosis. The different degrees of pancreatic necrosis in the patients with NP were as follows: 40 (26/14) patients with less than 30% necrosis, 80 (65/15) with 30%-50% necrosis, and 85 (72/13) with more than 50% necrosis. The median modified computer tomography severity scan (CTSI) score was 4 (range, 0-10). There was a significant difference in the degree of necrosis between the infection group and the non-infection group (Table 1).

Among the 205 NP patients, 179 were referred, with a median referral time of 24 d; 150 had infectious complications and 29 had aseptic NP. There was no significant difference in the referral time between the infection group and the non-infection group (Table 1).

Spectrum of pathogenic bacteria in infected patients

Among the 162 NP patients with confirmed infectious complications, 539 strains of pathogenic bacteria were isolated, of which 21 were cultured from 112 patients with pancreatic infection. The most common Gram-negative bacteria were *P. aeruginosa* and *E. coli* (n = 34). The most common Gram-positive bacterial species was *Enterococcus faecium* (n = 18), and the most common fungus was *Candida albicans* (n = 3). A total of 327 strains were cultured from 132 patients with extrapancreatic infection, including 165 strains from blood, 77 from the respiratory tract, 35 from the urinary tract, 25 from the abdominal cavity, 20 from the biliary tract, and 5 from wound infections. The most common Gram-negative bacterial species was *P. aeruginosa* (n = 32), the most common Gram-positive bacterial species was *Staphylococcus epidermidis* (n = 32), and the most common fungus was *C. albicans* (n = 19). Fungal infection was found in 39 patients; 4 strains of pathogenic fungi were cultured from three patients with pancreatic infections, and 52 strains of pathogenic fungi were cultured from 36 patients with extrapancreatic infections (24 from blood, 5 from the respiratory tract, and 17 from the urinary system) (Table 2).

Drug sensitivity analysis of pathogens in infected patients

Gram-negative bacteria: Almost all gram-negative bacteria cultured from cases of INP were resistant to first- and second-generation cephalosporins and nonenzymatic penicillin. The most common *E. coli* was sensitive to carbapenems and to third- and fourth-generation cephalosporins. *Klebsiella pneumoniae* was generally resistant to commonly used antibiotics, and the resistance rate to carbapenems was more than 50%. *P. aeruginosa* was more sensitive to third- and fourth-generation cephalosporins than to other antibiotic classes. The percentages of *E. coli* and *K. pneumoniae* strains producing extended-spectrum beta-lactamase (ESBL) were 41.18% (14/34) and 20% (6/30), respectively. The drug sensitivity of Gram-negative pathogenic bacteria in NP cases with extrapancreatic infection was low. *A. baumannii* and *K. pneumoniae* were the most sensitive of the identified species to tigecycline. *E. coli* and *P. aeruginosa* were still susceptible to cephalosporins and carbapenems (approximately 50%). The percentages of ESBL-positive *E. coli* and *K. pneumoniae* strains were both 50% (10/20) (Table 3).

Gram-positive bacteria: Gram-positive bacteria in INP patients were sensitive to vancomycin and tigecycline, but resistant to other antibiotics. The most common *E. faecium* was highly susceptible to macrolides (77.78%, 14/18). *Staphylococcus aureus* was almost 100% sensitive to quinolones, tetracyclines, penicillins, aminoglycosides, and sulfonamide compounds. The percentages of ESBL-positive *S. epidermidis* and *S. aureus* strains were 60% (3/5) and 60% (3/5), respectively. The drug sensitivities of Gram-positive pathogens in NP with extrapancreatic infection were slightly different. Most of these organisms were highly sensitive to macrolides, tetracyclines, and polypeptide antibiotics (> 80%). ESBL producing strains of *S. epidermidis* and *S. aureus* accounted for 37% (12/32) and 37.5% (3/8) of the strains, respectively (Table 4).

Fungal infection: According to the drug sensitivity of NP patients, *C. albicans* was the main pathogenic fungus cultured in patients with either pancreatic or extrapancreatic

Table 1 Comparison of baseline demographic and clinical characteristics between the infection group and non-infection group

Variable	Total (*n* = 205)	Infection (*n* = 163)	Non-infection (*n* = 42)	*P*-value
Age (yr)	49.5 ± 15.7	50.3 ± 15.5	46.6 ± 16.7	0.173
Male	140	112	28	0.801
Etiology				0.898
Biliary	109	88	21	
Alcohol	10	7	3	
Hyperlipemia	67	52	15	
ERCP	6	5	1	
Others	13	11	2	
Referral time	24 (0-300)	30 (0-300)	2.5 (0-180)	0.013[a]
CTSI	4 (2-10)	6 (2-10)	4 (2-8)	0.127
Persistent OF	50	49	1	0.005[b]
Need for any intervention	120	113	7	< 0.001[c]
Death	20	18	2	0.191
TPN	17 (0-163)	18 (0-163)	10 (0-47)	< 0.001[c]
EN	1 (0-112)	2 (0-112)	0 (0-34)	0.004[b]
Length of hospital stay(d)	29 (0-172)	32 (0-172)	19 (5-48)	< 0.001[c]
Length of stay ICU stay	11 (0-117)	13 (0-28)	7 (0-117)	0.004[b]
Necrosis degree				0.018[a]
< 30%	40	26	14	
30%-50%	80	65	15	
> 50%	85	72	13	

[a]$P < 0.05$,
[b]$P < 0.01$,
[c]$P < 0.001$. ERCP: Endoscopic retrograde cholangio pancreatography; CTSI: Computer tomography severity scan; OF: Organ failure; TPN: Total parenteral nutrition; EN: Enteral nutrition; ICU: Intensive care unit.

infection. *C. albicans* was sensitive to the antifungal drugs currently used in the clinic, such as 5-fluorocytosine, amphotericin B, and fluconazole.

MDR bacteria: Among the common Gram-negative bacilli identified in INP cases, the rates of multidrug resistance were as follows: *P. aeruginosa*, 26.47% (9/34); *K. pneumoniae*, 50% (15/30); *E. coli*, 26.47% (9/34); and *A. baumannii*, 72.73% (16/22). The multidrug resistance rate of common Gram-positive cocci was as follows: *E. faecium*, 27.78% (5/18). The multidrug resistance rates of common gram-negative bacteria in extrapancreatic infections were as follows: *P. aeruginosa*, 25% (8/32); *K. pneumoniae*, 68.75% (11/16); *E. coli*, 20% (4/20); and *A. baumannii*, 77.27% (17/22). The multidrug resistance rates of common gram-positive bacteria were as follows: *S. epidermidis*, 3.13% (1/32) and *E. faecium*, 13.33% (4/30).

Infection time and location

In this study, the extrapancreatic infection time was 9.1 ± 8.8 d, the pancreatic infection time was 13.9 ± 12.3 d, and the fungal infection time was (31.6 ± 26.4 d, $P < 0.05$). Among the 132 NP patients with extrapancreatic infection, 85 had bacteremia, 60 had respiratory tract infection, 25 had urinary tract infection, 22 had abdominal infection, 11 had biliary tract infection, and 4 had wound infection. The common sites of extrapancreatic infection in patients with early NP (< 14 d) were the blood and respiratory tract. The common sites of extrapancreatic infection in patients with advanced NP (> 14 d) were the blood, respiratory tract, and urinary tract (Figure 1).

Prognosis of NP patients

In this study, among the 42 cases of sterile NP, 35 were treated conservatively, and 7 were treated surgically. Six patients underwent intracystic drainage or video-assisted retroperitoneal debridement (VARD) to remove necrotic tissue. One patient was treated by interventional embolization for arterial hemorrhage. Among the 163 patients with infection, 50 received only symptomatic supportive treatment such as anti-inflammatory therapy, and 113 patients with NP underwent surgical treatment. Seven patients received only percutaneous catheter drainage (PCD) and anti-inflammatory treatment, while the remaining 106 patients received VARD and anti-

Table 2 Microbiological profile of organisms in infected patients

Organism	INP (*n* = 212)	Extrapancreatic infection (*n* = 327)
Gram-negative		
P. aeruginosa	34	32
A. baumanii	22	22
E. coli	34	20
K. pneumoniae	30	16
Gram-positive		
S. epidermidis	5	32
E. faecium	18	30
S. haemolyticus	5	19
S. aureus	5	8
Fungal		
C. albicans	3	19
C. parapsilosis	0	12
C. tropicalis	0	12
C. glabrata	1	9

INP: Infectious necrotizing pancreatitis; *E. coli*: *Escherichia coli*; *P. aeruginosa*: *Pseudomonas aeruginosa*; *A. baumannii*: *Acinetobacter baumannii*; *K. pneumoniae*: *Klebsiella pneumoniae*; *E. faecium*: *Enterococcus faecium*; *S. epidermidis*: *Staphylococcus epidermidis*; *S. aureus*: *Staphylococcus aureus*; *E. faecalis*: *Enterococcus faecalis*; *C. albicans*: *Candida albicans*; *C. parapsilosis*: *Candida parapsilosis*; *C. tropicalis*: *Candida tropicalis*; *C. glabrata*: *Candida glabrata*.

inflammatory treatment. Postoperative complications occurred in 21 patients: bleeding in 10, interventional therapy in 3 (hepatic artery in 1 and splenic artery in 2), digestive tract fistula in 8, and pancreatic fistula in 3.

In this study, the overall mortality rate was approximately 9.76% (*n* = 20). Of the 42 patients with sterile necrosis, 7 treated surgically were discharged from the hospital, and 2 treated conservatively died. Of the 163 NP patients with infectious complications, 7 died of infectious necrosis treated conservatively, and 2 died of PCD only. Nine patients died after VARD treatment. A total of 50 patients suffered from organ failure, including 1 in the noninfection group and 49 in the infection group. A total of 18 patients died, with a mortality rate of 36%. Regression analysis showed that the mortality rate was related to persistent organ failure, infection with multiple drug-resistant bacteria, and the number of operations (Table 5).

DISCUSSION

Many guidelines and studies suggest that infection is a risk factor for late mortality in NP patients and an indication for surgery[1-3,7-10]. Currently, the concept of surgery for INP patients has changed from "early, multiple, open debridement" to "delayed, drainage, minimally invasive". The superiority of the “step-up” approach to “open necrosectomy” for NP has been widely recognized[11-15]. Our center delays the progression of NP patients with suspected or confirmed infection by timely conservative treatment such as analgesia, fluid resuscitation, anti-inflammatory agents, and enteral (parenteral) nutrition. In a large number of clinical practices, we believe that the choice of antibiotic treatment for NP patients with infection, especially before the results of the first bacterial culture are obtained, is still relatively blind.

In our study, cultures of pathogenic bacteria from infected NP patients were analyzed. Gram-negative bacteria were the main pathogens in pancreatic and extrapancreatic infections. *E. coli* and *K. pneumoniae* were the most common pathogens in pancreatic infections, consistent with the findings in most studies on infectious pathogens[7,8,10,16-21]. Immune suppression in early NP patients may lead to excessive systemic inflammatory response syndrome, which increases intestinal mucosal permeability and damages intestinal commensal microorganisms, resulting in translocation of the intestinal flora and pancreatic infection. The main pathogenic bacteria in extrapancreatic infections were different from those in pancreatic infections. *P. aeruginosa* and *A. baumannii* were the most common Gram-negative bacteria in extrapancreatic infection. *E. coli* and *K. pneum oniae* were the main Gram-negative bacteria in pancreatic infections. The bacteria in pancreatic infections may

Table 3 Drug sensitivity of Gram-negative bacteria in infected patients

	Pancreatic				Extrapancreatic			
Antibiotic	*P. aeruginosa*	*E. coli*	*A. baumannii*	*K. pneumoniae*	*P. aeruginosa*	*E. coli*	*A. baumannii*	*K. pneumoniae*
MDR bacteria	9/34	9/34	16/22	15/30	8/32	4/20	17/22	11/16
ESBL +:	–	14/34	–	6/30	–	10/20	–	2/16
Penicillins								
Ampicillin	0/34	8/34	0/22	1/30	0/32	4/20	1/22	0/16
Piperacillin	12/34	4/34	0/22	3/30	14/32	4/20	1/22	0/16
Enzymatic penicillins								
Piperacillin/tazobactam	13/34	10/34	4/22	8/30	19/32	11/20	1/22	4/16
Cefoperazone/sulbactam	8/34	0/34	4/22	0/30	9/32	0/20	4/22	0/16
Ampicillin/sulbactam	0/34	4/34	0/22	4/30	0/32	6/20	4/22	0/16
quinolones								
Levofloxacin	21/34	11/34	3/22	11/30	17/32	6/20	3/22	3/16
First- and second-generation cephalosporins								
Cefoxitin	0/34	0/34	0/22	0/30	0/32	0/20	0/22	0/16
Cefuroxime	0/34	0/34	0/22	0/30	0/32	0/20	0/22	0/16
Third- and fourth-generation cephalosporins								
Ceftazidime	14/34	13/34	3/22	7/30	16/32	11/20	3/22	2/16
Cefepime	17/34	12/34	3/22	10/30	15/32	11/20	3/22	3/16
Carbapenems								
Imipenem	10/34	21/34	2/22	11/30	10/32	12/20	3/22	2/16
Meropenem	10/34	11/34	3/22	6/30	9/32	6/20	3/22	2/16
Sulfonamides								
Compound trimethoprim	1/34	14/34	9/22	14/30	0/32	10/20	8/22	9/16
Tetracycline								
Tigecycline	1/34	1/34	17/22	13/30	0/32	2/20	18/22	9/16
Minocycline	–	–	9/22	–	0/32	–	10/22	–

MDR: Multidrug-resistant; ESBL: Extended-spectrum β-lactamases; *E. coli*: *Escherichia coli*; *P. aeruginosa*: *Pseudomonas aeruginosa*; *A. baumannii: Acinetobacter baumannii*; *K. pneumoniae*: *Klebsiella pneumoniae*.

originate primarily from the intestinal tract; thus, intestinal bacteria are common, while the bacteria in extrapancreatic infections differ by infection site.

According to the analysis of extrapancreatic infection time in NP patients, the extrapancreatic infection time was earlier than the pancreatic infection time, consistent with the conclusions of other studies[4,5,7,8]. In both early (< 14 d) and late (> 42 d) infections, bacteremia is a common source of infection. In the early stage, bacteremia may occur due to the suppression of immune function by a systemic inflammatory reaction, which may affect the prognosis of NP patients[5]. Brown *et al*[4] noted that the most common manifestations of extrapancreatic infections in severe AP patients were respiratory tract infections (9.2%) and bacteremia (8.4%). Moka *et al*[8] noted that INP patients with extrapancreatic infection (pneumonia or bacteremia) had significantly higher total hospitalization lengths, ICU hospitalization lengths, and mortality rates than those without extrapancreatic infection. In a retrospective study by Besselink *et al*[10], bacteremia was considered an independent risk factor for predicting death in AP patients, and increased the risk of pancreatic parenchymal necrosis and infection. The predominance of bacteremia in late extrapancreatic infections may be related to long hospitalization times, multiple operations, secondary iatrogenic infections, or prolonged indwelling times of deep venous catheters and catheters for long-term parenteral nutrition[22].

In this study, the multidrug resistance rate of common Gram-negative bacteria (*K. pneumoniae*, *P. aeruginosa*, *E. coli*, and *A. baumannii*) found in INP in our center ranged from 23.68% to 72.73%. The multidrug resistance rate of common Gram-positive bacteria (*E. faecium*) was 27.78%. The multidrug resistance rates of common Gram-negative bacteria (*K. pneumoniae*, *P. aeruginosa*, *E. coli*, and *A. baumannii*) in extrapancreatic infections ranged from 25% to 81.82%. The multidrug resistance rate of common G ram-positive bacteria (*E. faecium*) was 20%. Mourad *et al*[21] have also reported that prolonged antibiotic use leads to MDR bacterial and fungal infections,

Table 4 Drug sensitivity of Gram-positive bacteria in infected patients

	Pancreatic				Extrapancreatic			
Antibiotic	*E. faecium*	*S. epidermidis*	*S. aureus*	*E. faecalis*	*E. faecium*	*S. epidermidis*	*S. aureus*	*E. faecalis*
MDR bacteria	5/18	0/5	2/5	3/6	4/30	1/32	3/8	0/5
ESBL +:	–	3/5	3/5	–	–	12/32	3/8	–
Penicillins								
Ampicillin	2/18	0/5	0/5	3/6	1/30	0/32	0/8	3/5
Penicillin G	0/18	0/5	0/5	1/6	0/30	0/32	0/8	3/5
quinolones								
Ciprofloxacin	1/18	0/5	0/5	1/6	0/30	8/32	3/8	3/5
Levofloxacin	2/18	0/5	0/5	1/6	1/30	8/32	3/8	3/5
Moxifloxacin	1/18	2/5	0/5	1/6	1/30	21/32	3/8	3/5
Macrolactones								
Linezolid	14/18	5/5	4/5	5/6	25/30	31/32	8/8	4/5
Tetracycline								
Tigecycline	17/18	5/5	5/5	5/6	27/30	32/32	8/8	5/5
Sulfonamides								
Compound trimethoprim	1/18	1/5	4/5	0/6	2/30	9/32	6/8	2/5
Polypeptides								
Vancomycin	11/18	5/5	5/5	6/6	25/30	31/32	8/8	5/5

MDR: Multidrug-resistant; ESBL: Extended-spectrum β-lactamase; *E. faecium*: *Enterococcus faecium*; *S. epidermidis*: *Staphylococcus epidermidis*; *S. aureus*: *Staphylococcus aureus*; *E. faecalis*: *Enterococcus faecalis*.

which are also associated with prolonged hospitalization and poor prognosis. Therefore, it is necessary to replace antibiotics to which pathogens are sensitive or discontinue drugs in a timely manner, according to the patient's condition, to reduce the risk of the bacteria developing multidrug resistance. In this study, 39 patients developed fungal infections and 56 strains of pathogenic fungi were cultured. The most common pathogenic fungus was *C. albicans*. Notably, the 39 cases of fungal infection were all mixed infections, and the occurrence time of fungal infections was later than that of bacterial infections. This difference may be related to the long-term use of broad-spectrum antibiotics and the decline of autoimmunity in NP patients. Most studies consider fungal infection a risk factor for mortality in infected patients[7,8,10,19,23-25]. However, whether antifungal drugs should be used prophylactically in patients with suspected or confirmed extrapancreatic (and) or pancreatic infections requiring antibiotics remains controversial. The preventive use of broad-spectrum antibiotics or antifungal therapy may increase the incidence of bacterial or fungal superinfection[8,19]. The preventive use of antibiotics has not increased the incidence of fungal infections and may reduce the incidence of fungal infections in patients, but further large-scale studies are needed to confirm this hypothesis[23,24]. In this study, the degree of pancreatic necrosis, CTSI score, rate of referral, rate of organ failure, need for surgical intervention, need for enteral nutrition, need for parenteral nutrition, and length of hospitalization were higher in NP patients in the infection group than in NP patients in the non-infection group. In the infection group, most patients (n = 150) were referred from an outside hospital. Their basic vital signs and immune function were poor at admission, and the incidence of adverse events such as organ failure and aggravation of pancreatic necrosis increased with prolonged disease course. Long-term nutritional support and even surgery were needed, which prolonged the hospitalization time of the patients. In addition, many studies support the view that the risk of pancreatic infection increases with the degree of pancreatic necrosis[1-3,11-16]. However, in this study, the infection rate of NP patients with simple extrapancreatic infection was also higher than that of the non-infection group, which may be related to the poor autoimmunity and severe inflammation in patients with extrapancreatic infection. In addition, false negative results for the culture of pathogenic bacteria from the pancreas in some patients with simple extrapancreatic infection were not excluded.

In addition, some issues need to be addressed. First, most patients were treated with antibiotics before surgery, and the related effects need to be further analyzed. Second, a few patients were diagnosed with INP during outpatient treatment, but there was no detailed record of the infectious strains, and there was a certain

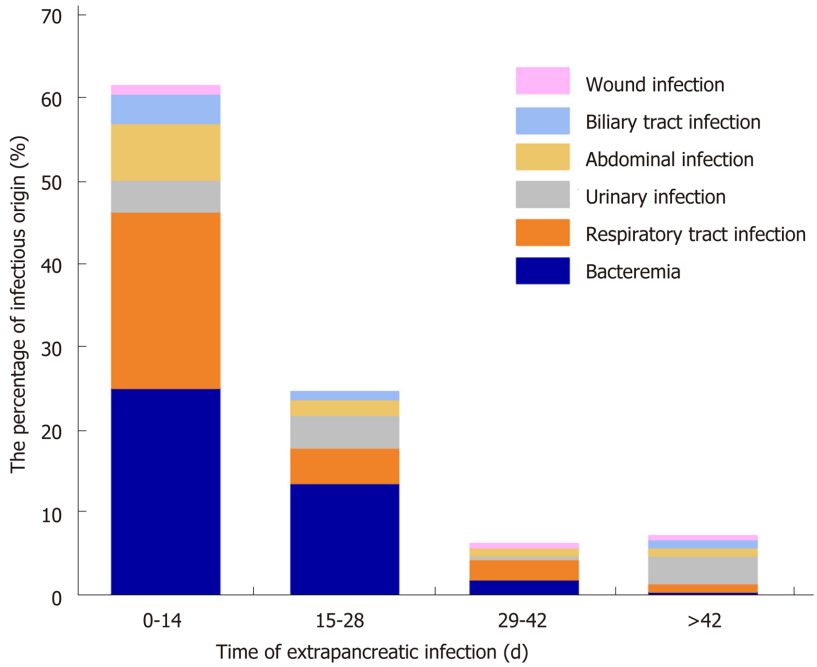

Figure 1 Time and location distribution of extrapancreatic infection. In the early stage of necrotizing pancreatitis (NP), the most common sites of extrapancreatic infection were the blood, respiratory tract, and abdominal cavity. In the late stage of NP, the most common sites of extrapancreatic infection were the blood, respiratory tract, and urinary tract. NP: Necrotizing pancreatitis.

deviation in the number of pathogenic bacteria. Third, the changes in pathogenic bacteria before and after surgery were not analyzed. Finally, the difference between pancreatic necrosis and peri-pancreatic necrosis was not mentioned.

In conclusion, when NP patients present clinical symptoms of suspected infection, such as fever, elevated inflammatory indexes, and sepsis, but no clear indication of pancreatic infection revealed by enhanced CT examination, the culture from site of extrapancreatic infection can be given priority (different extrapancreatic culture sites can be selected according to the onset time). If no extrapancreatic infection was diagnosed, surgical procedures should be considered to determine whether the patient has pancreatic infection. In addition, our study has shown that although Gram-negative bacteria are still dominant in NP patients, MDR bacterial infection tends to increase. Therefore, anti-infection treatment should be carried out after it is clear that the patient has symptoms of infection. For NP patients with confirmed infection, priority can be given to antibiotics to which Gram-negative bacteria are sensitive, and then sensitive antibiotics can be selected according to the results of drug sensitivity. When NP patients are clearly complicated with infection symptoms, they should be paid close attention to the risk of organ failure, MDR bacterial infection, or multiple operations. Hence, understanding the pathogen spectrum and drug sensitivity testing of NP patients with infectious symptoms will help clinicians to use antibiotics effectively and reasonably, improve the therapeutic effect, and reduce the related mortality of NP patients.

Table 5 Multivariable analysis for independent predictors of mortality

Variable	Univariable analysis	*P*-value	Multivariable analysis	*P*-value
	OR (95%CI)		OR (95%CI)	
Persistent OF	5.37 (3.785-7.618)	< 0.001	0.012 (0.001-0.101)	< 0.001[c]
MDR organism (s)	2.445 (1.554-3.848)	0.001	0.134 (0.028-0.633)	0.011[b]
Fungal infection	2.719 (1.498-4.936)	0.002	0.657 (0.156-2.770)	0.567
Prophylactic antibiotic	0.974 (0.783-1.210)	0.8	0.534 (0.095-2.989)	0.475
INP	1.151 (0.802-1.651)	0.48	0.277 (0.024-3.234)	0.306
Extrapancreatic infection	1.383 (1.124-1.704)	0.031	0.627 (0.042-9.273)	0.734
Referred patients	1.102 (0.986-1.232)	0.25	0.524 (0.041-6.655)	0.619
Operation	0.789 (0.497-1.255)	0.262	10.533 (1.052-105.491)	0.045[a]
Complication	2.921 (1.304-6.540)	0.011	0.892 (0.191-4.162)	0.885

[a]$P < 0.05$,
[b]$P < 0.01$,
[c]$P < 0.001$. OF: Organ failure; INP: Infectious necrotizing pancreatitis; TPN: Total parenteral nutrition; EN: Enteral nutrition; CI: Confidence interval; OR: Odds ratio; MDR: Multidrug-resistant.

ARTICLE HIGHLIGHTS

Research background

As acute pancreatitis patients progress to necrotizing pancreatitis (NP), their hospitalization costs and mortality rates become much higher than those of patients with edematous pancreatitis. Approximately 40%-70% of NP cases present with symptoms of infection, and infection has become the most important risk factor for death from NP. Therefore, it is critical to identify patients with NP who have symptoms of infection.

Research motivation

Studies on pancreatic infectious have mainly focused on pancreatic and peripancreatic infections, and little is known about extrapancreatic infections and coinfections. We considered the difference in infection time, infection site, and infectious strains in NP patients with infectious complications, and found that early intervention may decrease the mortality rate.

Research objectives

Infectious NP, extrapancreatic infection, and coinfection are the common complications of NP. The research is critical to improve the treatment strategy for NP patients with infectious complications.

Research methods

This study included 205 patients with NP. The baseline data were recorded as the mean ± SD or medians (ranges) as appropriate. Independent samples *t*-tests were performed to analyze the differences between the groups. Qualitative data were scored, and chi-square or Fisher's exact tests were performed to analyze the differences between the groups. Logistic regression was used to determine the risk of death in the patients using *via* SPSS 23.0 statistical software.

Research results

A total of 539 strains of pathogenic bacteria were cultured from the 162 NP patients with confirmed infectious complications. The most common Gram-negative bacteria were *P. aeruginosa* and *E. coli* ($n = 34$). The most common Gram-positive bacteria were *E. faecium* ($n = 18$), and the most common fungus was *C. albicans* ($n = 3$). The extrapancreatic infection time was 9.1 ± 8.8 d, the pancreatic infection time was 13.9 ± 12.3 d, and the fungal infection time was 31.6 ± 26.4 d ($P < 0.05$). The common sites of extrapancreatic infection in patients with advanced NP (> 14 d) were the blood, respiratory tract, and urinary tract.

Research conclusions

This retrospective analysis of NP patients with infectious com-plications in our hospital shows that the main pathogenic bacteria in NP patients were Gram-negative bacteria, the most common of which were *E. coli* and *P. aeruginosa*. Additionally, it was found that the proportion of multidrug-resistant bacteria was relatively large, and caution should be used in the application of antibiotics. The extrapancreatic infection time was usually earlier than the pancreatic infection time. For patients with suspected extrapancreatic infection, blood and respiratory pathogen culture should be prioritized. Third- or fourth-generation cephalosporins or carbapenems can be used as empirical antibiotics. Persistent organ failure, multidrug resistance, and surgery were risk factors for death.

Research perspectives

We confirmed the common infectious strains, the site of infection, and the time of infection in NP patients with infectious complications. Through these results, we found that it is helpful for clinicians to evaluate the patient's condition in a timely manner to improve the patient's prognosis.

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REFERENCES

1 **van Dijk SM**, Hallensleben NDL, van Santvoort HC, Fockens P, van Goor H, Bruno MJ, Besselink MG; Dutch Pancreatitis Study Group. Acute pancreatitis: recent advances through randomised trials. *Gut* 2017; **66**: 2024-2032 [PMID: 28838972 DOI: 10.1136/gutjnl-2016-313595]

2 **Banks PA**, Bollen TL, Dervenis C, Gooszen HG, Johnson CD, Sarr MG, Tsiotos GG, Vege SS; Acute Pancreatitis Classification Working Group. Classification of acute pancreatitis--2012: revision of the Atlanta classification and definitions by international consensus. *Gut* 2013; **62**: 102-111 [PMID: 23100216 DOI: 10.1136/gutjnl-2012-302779]

3 **Werge M**, Novovic S, Schmidt PN, Gluud LL. Infection increases mortality in necrotizing pancreatitis: A systematic review and meta-analysis. *Pancreatology* 2016; **16**: 698-707 [PMID: 27449605 DOI: 10.1016/j.pan.2016.07.004]

4 **Brown LA**, Hore TA, Phillips AR, Windsor JA, Petrov MS. A systematic review of the extra-pancreatic infectious complications in acute pancreatitis. *Pancreatology* 2014; **14**: 436-443 [PMID: 25455539 DOI: 10.1016/j.pan.2014.09.010]

5 **Pando E**, Alberti P, Hidalgo J, Vidal L, Dopazo C, Caralt M, Blanco L, Gómez-Gavara C, Bilbao I, Balsells J, Charco R. The role of extra-pancreatic infections in the prediction of severity and local complications in acute pancreatitis. *Pancreatology* 2018 [PMID: 29802078 DOI: 10.1016/j.pan.2018.05.481]

6 **Balthazar EJ**, Ranson JH, Naidich DP, Megibow AJ, Caccavale R, Cooper MM. Acute pancreatitis: prognostic value of CT. *Radiology* 1985; **156**: 767-772 [PMID: 4023241 DOI: 10.1148/radiology.156.3.4023241]

7 **Jain S**, Mahapatra SJ, Gupta S, Shalimar, Garg PK. Infected Pancreatic Necrosis due to Multidrug-Resistant Organisms and Persistent Organ failure Predict Mortality in Acute Pancreatitis. *Clin Transl Gastroenterol* 2018; **9**: 190 [PMID: 30287818 DOI: 10.1038/s41424-018-0056-x]

8 **Moka P**, Goswami P, Kapil A, Xess I, Sreenivas V, Saraya A. Impact of Antibiotic-Resistant Bacterial and Fungal Infections in Outcome of Acute Pancreatitis. *Pancreas* 2018; **47**: 489-494 [PMID: 29517630 DOI: 10.1097/MPA.0000000000001019]

9 **Bohidar NP**, Garg PK, Khanna S, Tandon RK. Incidence, etiology, and impact of Fever in patients with acute pancreatitis. *Pancreatology* 2003; **3**: 9-13 [PMID: 12649559 DOI: 10.1159/000069146]

10 **Besselink MG**, van Santvoort HC, Boermeester MA, Nieuwenhuijs VB, van Goor H, Dejong CH, Schaapherder AF, Gooszen HG; Dutch Acute Pancreatitis Study Group. Timing and impact of infections in acute pancreatitis. *Br J Surg* 2009; **96**: 267-273 [PMID: 19125434 DOI: 10.1002/bjs.6447]

11 **Rao C**, Bhasin DK, Rana SS, Gupta R, Gautam V, Singh K. Implications of culture positivity in acute pancreatitis: does the source matter? *J Gastroenterol Hepatol* 2013; **28**: 887-892 [PMID: 23425090 DOI: 10.1111/jgh.12161]

12 **Li A**, Cao F, Li J, Fang Y, Wang X, Liu DG, Li F. Step-up mini-invasive surgery for infected pancreatic necrosis: Results from prospective cohort study. *Pancreatology* 2016; **16**: 508-514 [PMID: 27083075 DOI: 10.1016/j.pan.2016.03.014]

13 **van Santvoort HC**, Besselink MG, Bakker OJ, Hofker HS, Boermeester MA, Dejong CH, van Goor H, Schaapherder AF, van Eijck CH, Bollen TL, van Ramshorst B, Nieuwenhuijs VB, Timmer R, Laméris JS, Kruyt PM, Manusama ER, van der Harst E, van der Schelling GP, Karsten T, Hesselink EJ, van Laarhoven CJ, Rosman C, Bosscha K, de Wit RJ, Houdijk AP, van Leeuwen MS, Buskens E, Gooszen HG; Dutch Pancreatitis Study Group. A step-up approach or open necrosectomy for necrotizing pancreatitis. *N Engl J Med* 2010; **362**: 1491-1502 [PMID: 20410514 DOI: 10.1056/NEJMoa0908821]

14 **Horvath K**, Freeny P, Escallon J, Heagerty P, Comstock B, Glickerman DJ, Bulger E, Sinanan M, Langdale L, Kolokythas O, Andrews RT. Safety and efficacy of video-assisted retroperitoneal debridement for infected pancreatic collections: a multicenter, prospective, single-arm phase 2 study. *Arch Surg* 2010; **145**: 817-825 [PMID: 20855750 DOI: 10.1001/archsurg.2010.178]

15 **Hollemans RA**, Bakker OJ, Boermeester MA, Bollen TL, Bosscha K, Bruno MJ, Buskens E, Dejong CH, van Duijvendijk P, van Eijck CH, Fockens P, van Goor H, van Grevenstein WM, van der Harst E, Heisterkamp J, Hesselink EJ, Hofker S, Houdijk AP, Karsten T, Kruyt PM, van Laarhoven CJ, Laméris JS, van Leeuwen MS, Manusama ER, Molenaar IQ, Nieuwenhuijs VB, van Ramshorst B, Roos D, Rosman C, Schaapherder AF, van der Schelling GP, Timmer R, Verdonk RC, de Wit RJ, Gooszen HG, Besselink MG, van Santvoort HC; Dutch Pancreatitis Study Group. Superiority of Step-up Approach vs Open Necrosectomy in Long-term Follow-up of Patients With Necrotizing Pancreatitis. *Gastroenterology* 2019; **156**: 1016-1026 [PMID: 30391468 DOI: 10.1053/j.gastro.2018.10.045]

16 **Kokosis G**, Perez A, Pappas TN. Surgical management of necrotizing pancreatitis: an overview. *World J Gastroenterol* 2014; **20**: 16106-16112 [PMID: 25473162 DOI: 10.3748/wjg.v20.i43.16106]

17 **Mowbray NG**, Ben-Ismaeil B, Hammoda M, Shingler G, Al-Sarireh B. The microbiology of infected pancreatic necrosis. *Hepatobiliary Pancreat Dis Int* 2018; **17**: 456-460 [PMID: 30197163 DOI: 10.1016/j.hbpd.2018.08.007]

18 **Behrman SW**, Bahr MH, Dickson PV, Zarzaur BL. The microbiology of secondary and postoperative pancreatic infections: implications for antimicrobial management. *Arch Surg* 2011; **146**: 613-619 [PMID:

21576614 DOI: 10.1001/archsurg.2011.85]
19 **Schmidt PN**, Roug S, Hansen EF, Knudsen JD, Novovic S. Spectrum of microorganisms in infected walled-off pancreatic necrosis - impact on organ failure and mortality. *Pancreatology* 2014; **14**: 444-449 [PMID: 25266641 DOI: 10.1016/j.pan.2014.09.001]
20 **Shen Y**, Cui NQ. Clinical observation of immunity in patients with secondary infection from severe acute pancreatitis. *Inflamm Res* 2012; **61**: 743-748 [PMID: 22466614 DOI: 10.1007/s00011-012-0467-1]
21 **Mourad MM**, Evans R, Kalidindi V, Navaratnam R, Dvorkin L, Bramhall SR. Prophylactic antibiotics in acute pancreatitis: endless debate. *Ann R Coll Surg Engl* 2017; **99**: 107-112 [PMID: 27917667 DOI: 10.1308/rcsann.2016.0355]
22 **Wu BU**, Johannes RS, Kurtz S, Banks PA. The impact of hospital-acquired infection on outcome in acute pancreatitis. *Gastroenterology* 2008; **135**: 816-820 [PMID: 18616944 DOI: 10.1053/j.gastro.2008.05.053]
23 **Kochhar R**, Noor MT, Wig J. Fungal infections in severe acute pancreatitis. *J Gastroenterol Hepatol* 2011; **26**: 952-959 [PMID: 21299617 DOI: 10.1111/j.1440-1746.2011.06685.x]
24 **Trikudanathan G**, Navaneethan U, Vege SS. Intra-abdominal fungal infections complicating acute pancreatitis: a review. *Am J Gastroenterol* 2011; **106**: 1188-1192 [PMID: 21731015 DOI: 10.1038/ajg.2010.497]
25 **Shen D**, Wang D, Ning C, Lin C, Cao X, Liu Z, Ji L, Huang G. Prognostic factors of critical acute pancreatitis: A prospective cohort study. *Dig Liver Dis* 2019 [PMID: 31079936 DOI: 10.1016/j.dld.2019.04.007]

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ORIGINAL ARTICLE

Retrospective Study

Risk factors for Mallory-Weiss Tear during endoscopic submucosal dissection of superficial esophageal neoplasms

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Abstract

BACKGROUND

Adverse events during endoscopic submucosal dissection (ESD) of superficial esophageal neoplasms, such as perforation and bleeding, have been well-documented. However, the Mallory-Weiss Tear (MWT) during esophageal ESD remains under investigation.

AIM

To investigate the incidence and risk factors of the MWT during esophageal ESD.

METHODS

From June 2014 to July 2017, patients with superficial esophageal neoplasms who received ESD in our institution were retrospectively analyzed. The clinicopathological characteristics of the patients were collected. Patients were divided into an MWT group and non-MWT group based on whether MWT occurred during ESD. The incidence of MWTs was determined, and the risk factors for MWT were then further explored.

RESULTS

A total of 337 patients with 373 lesions treated by ESD were analyzed. Twenty patients developed MWTs during ESD (5.4%). Multivariate analysis identified that female sex (OR = 5.36, 95%CI: 1.47-19.50, P = 0.011) and procedure time longer than 88.5 min (OR = 3.953, 95%CI: 1.497-10.417, P = 0.005) were independent risk factors for an MWT during ESD. The cutoff value of the procedure time for an MWT was 88.5 min (sensitivity, 65.0%; specificity, 70.8%). Seven of the MWT patients received endoscopic hemostasis. All patients recovered satisfactorily without surgery for the laceration.

CONCLUSION

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The incidence of MWTs during esophageal ESD was much higher than expected. Although most cases have a benign course, fatal conditions may occur. We recommend inspection of the stomach during and after the ESD procedure for timely management in cases of bleeding MWTs or even perforation outside of the procedure region.

Key words: Superficial esophageal neoplasms; Endoscopic submucosal dissection; Mallory-Weiss Tear; Incidence; Risk factors

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Core tip: To our knowledge, no literature has focused on the risk factors for an Mallory-Weiss Tear (MWT) during esophageal endoscopic submucosal dissection (ESD). Thus, the present study aimed to clarify the incidence of WMTs during esophageal ESD, and to evaluate associated risk factors. In this work, we found that female sex and procedure time were independent risk factors for an MWT during ESD. For patients with such characteristics, clinicians must remain vigilant and perform careful observations after ESD.

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INTRODUCTION

Endoscopic submucosal dissection (ESD) has been widely accepted as an effective treatment for superficial esophageal neoplasms when the risk of lymph node metastasis is diagnosed as being very low or negligible[1-3]. Adverse events, such as bleeding, muscularis propria injury, and even perforation during the procedure are well-documented by endoscopists, and can mostly be managed endoscopically[3-5]. However, the Mallory-Weiss Tear (MWT) during ESD is not well-understood.

The MWT, first described in 1929, is characterized by a linear, non-perforation mucosal laceration at the gastroesophageal junction that is induced by vomiting and retching[6]. Previous studies have reported that the classical MWT (non-iatrogenic MWT) accounted for 7%-14% of acute upper gastrointestinal bleeding, including even cirrhotic patients[7-10]. The first documented case of an MWT resulting from endoscopy was reported by Watts in 1976[11], and the incidence was reported to be 0.08%-0.49%[12-15]. Associated risk factors for an MWT during screening endoscopy include hiatal hernia, female sex, a history of distal gastrectomy and old age[14-16]. With the development of endoscopic technology and growing emphasis on the diagnosis and endoscopic treatment of early esophageal cancer, ESD-associated MWT is reported case by case. However, to our knowledge, no literature has focused on the risk factors for an MWT during esophageal ESD. Thus, the present study aimed to clarify the incidence of WMTs during esophageal ESD, and to evaluate the associated risk factors.

MATERIALS AND METHODS

Patients

From June 2014 to July 2017, 341 consecutive patients with 377 superficial esophageal neoplasms were treated by ESD at the West China Hospital of Sichuan University. The indication criteria of ESD for superficial esophageal neoplasms were: (1) Intraepithelial neoplasia or carcinoma determined by biopsy, performed during chromoendoscopy with iodine staining; (2) Depth of invasion limited to within sm1, as assessed by endoscopic ultrasonography (EUS) and/or magnifying endoscopy with narrow-band imaging (ME-NBI); and (3) The absence of lymph node or distant metastasis confirmed by contrast-enhanced computed tomography (CT) of the chest and abdomen. Patients with a history of esophagectomy, proximal gastrectomy or

total gastrectomy were excluded from this study. Additionally, all patients completed diagnostic endoscopies before treatment without an MWT occurring. Written informed consent was obtained from all patients before the procedures. The present study was reviewed and approved by the Ethics Committee of the West China Hospital of Sichuan University.

ESD procedure and post-ESD management

The ESD procedure was categorized as conventional ESD and endoscopic submucosal tunnel dissection (ESTD) (Figure 1). The conventional ESD procedure comprised four steps: (1) Marking the margin of the lesion; (2) Submucosal injection to elevate the lesion; (3) Mucosal incision around the lesion; and (4) Submucosal dissection. Regarding ESTD, one or more submucosal tunnels were established during the dissection step. The ESD procedure was decided by the endoscopist according to the size of the lesion.

ESD was performed with a single-accessory channel endoscope (GIF-Q260J, Olympus, Tokyo, Japan) with a transparent cap (D-201-10704, Olympus, Tokyo, Japan) attached to the front. Other equipment and accessories included: A high-frequency generator (VIO200D; ERBE ELEKTROMEDIZIN GMBH, Germany), argon plasma coagulation (APC) unit (APC-ICC200; ERBE ELEKTROMEDIZIN GMBH, Germany), IT knife (KD -611L, Olympus, Tokyo, Japan), Dual-knife (KD-650L/Q, Olympus, Tokyo, Japan), injection needle (NM-200U-0423, Olympus, Tokyo, Japan), and hemostatic forceps (FD-410 LR, Olympus, Tokyo, Japan).

All patients were under mechanically-ventilated general anesthesia, and in the supine position. ESD was performed by one experienced endoscopist (with 25 years of endoscopy experience and > 300 cases of esophageal ESD) and two less-experienced endoscopists (with 15 years of endoscopy experience and approximately 100 cases of esophageal ESD), and carbon dioxide was used for insufflation. After resection of the esophageal lesion, the gastrointestinal decompression tube was placed, and proton-pump inhibitors (PPI) and hemostatic drugs were used on all patients. Endoscopic follow up was scheduled at 1, 3, 6, and 12 mo after ESD, and then annually.

Treatment for the MWT

The decisions regarding the treatment strategy, including whether to treat the MWT endoscopically, were made by endoscopists according to the size of the laceration and the presence of active bleeding. Based on the endoscopic findings, APC, hemoclips or hemoclips combined with endoloop were used to treat the MWT.

Definitions

MWT was defined as a mucosal tear at the esophagastric junction with or without active bleeding during the ESD procedure. Atrophic gastritis and hiatal hernia were diagnosed based on endoscopic findings. The longitudinal diameter of the esophageal lesion was measured from the oral edge to the anal edge of the resected specimens, and the circumferential diameter was determined based on the length of the vertical axis against the longitudinal axis. The circumferential extent of the mucosal defect was judged by the endoscopists. Curative resection was defined as *en bloc* resection with tumor-free horizontal and vertical margins, and met the following conditions simultaneously: Invasion depth limited in the submucosa < 200 μm without lymphovascular involvement.

Data analysis

Data pertaining to potential predisposing factors, such as age, sex, atrophic gastritis, hiatal hernia, characteristics of the esophageal lesions, and the experience of the endoscopists were collected from our prospectively maintained endoscopic database.

Variables are reported using the median (range) and simple proportion according to the type of data. The Mann-Whitney *U* test, chi-square test or Fisher's exact test were used for univariate analysis, as appropriate. Clinically or statistically significant variables in the univariate analysis were included in the multivariate logistic regression analysis to assess the independent risk factors for an MWT. Differences with a $P < 0.05$ were considered significant. Statistical analysis was performed using SPSS version 23 for Windows.

RESULTS

A total of 341 patients with 377 superficial esophageal neoplasms were treated by ESD from June 2014 to July 2017 in our institution. Four patients with four lesions were excluded because of a history of esophagectomy, proximal gastrectomy or total gastrectomy. The remaining 337 patients with 373 lesions were reviewed for the

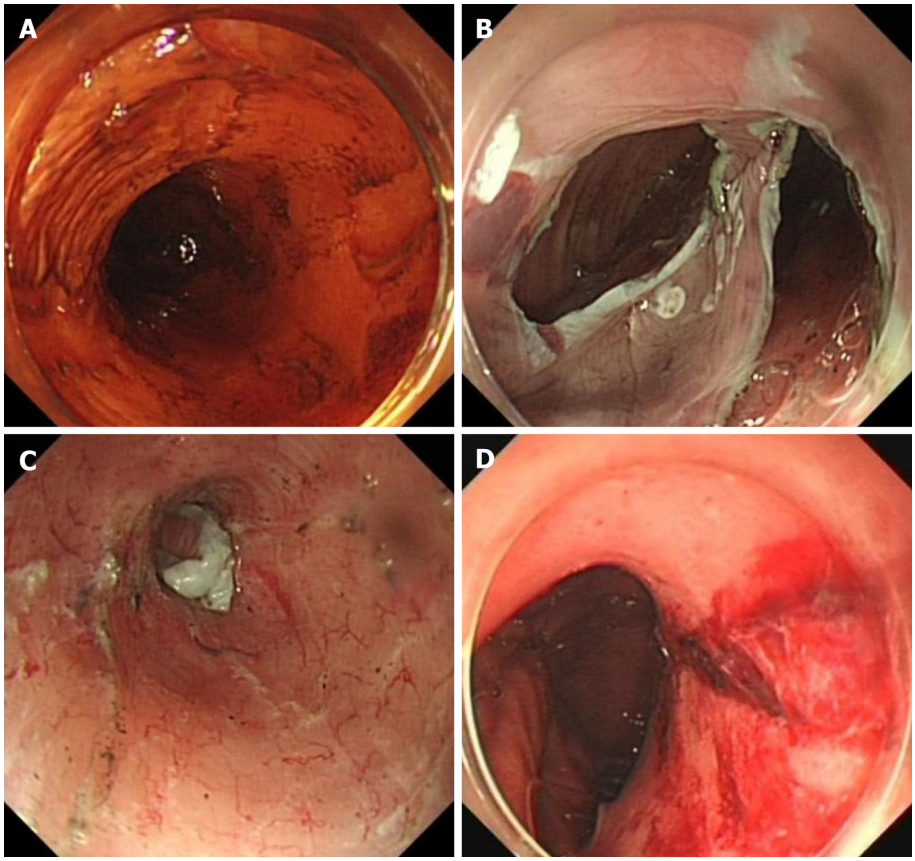

Figure 1 Mallory-Weiss Tear occurred during ESTD. A: The lesion of the esophagus after iodine staining; B: The submucosal tunnel created during ESTD; C: The artificial wound after ESTD; D: The mucosal laceration at the gastro-esophageal junction. ESTD: Endoscopic submucosal tunnel dissection.

present study. During the period of study, 20 (5.4%) MWT cases developed during the ESD procedure.

Characteristics of all patients and lesions

The clinicopathological features of the patents and lesions are shown in Table 1. Overall, the median age of the patients was 62 years (range, 37-83 years), and the male proportion was 67.7%. Of the 373 lesions, 67.6% (252/373) were located in the middle esophagus, and 26.8% (100/373) were located in the lower esophagus. The median circumferential and longitudinal diameters of the specimen were 2.5 cm (range, 0.5-7.0 cm) and 4.0 cm (range, 1.0-14.0 cm), respectively. The median procedure time was 60 min (range, 12-240 min). The *en bloc* resection rate and curative resection rate were achieved in 96.2% (359/373) and 86.1% (321/373) of the lesions, respectively. The histology and invasion depth were as follows: Intraepithelial neoplasia, 131 (35.1%); mucosal invasion, 194 (52.0%); SM < 200 μm invasion, 21 (5.6%); and SM ≥ 200 μm invasion 26 (7.0%). Perforations developed in 6 (1.6%) cases, and the incidence of stenosis was 12.3% (46/373).

Incidence and risk factors for an MWT

Twenty patients developed MWTs during the ESD procedure. The characteristics of the patients with or without MWTs are shown in Table 2. Predisposing factors[14,16] including atrophic gastritis, hiatal hernia, and a history of distal gastrectomy were not observed in the MWT cases. There was no significant difference in age, concomitant diseases, location, size, invasion depth, type of resection, or experience of the endoscopists between the two groups. However, in the MWT group, the proportion of female patients was much higher (70.0% *vs* 30.0%, $P < 0.001$), and the procedure time was longer (90 *vs* 58 min, $P = 0.008$) than the non-MWT group. Multivariate logistic regression analysis (Table 3) revealed that female sex was an independent risk factor for an MWT during esophageal endoscopic resection (OR = 5.27, 95%CI: 1.94-14.32, $P = 0.001$), and the procedure time longer than 88.5 mins was also an independent risk factor for an MWT during esophageal endoscopic resection (OR = 3.953, 95%CI: 1.497-10.417, $P = 0.005$). The association between MWT and procedure time was evaluated using receiver operating characteristic (ROC) curve analysis, and the cutoff value of

Table 1 Clinicopathological features of all patients and lesions

Characteristics	*n* (%)/median (range)
No. of patients	337
Age in yr	62 (37-83)
Sex	
Male	228 (67.7)
Female	109 (32.3)
Concomitant diseases	
Atrophic gastritis	15 (4.4)
Hiatal hernia	2 (0.6)
History of distal gastrectomy	4 (1.2)
No. of lesions	373
Location	
Upper	21 (5.6)
Middle	252 (67.6)
Lower	100 (26.8)
Longitudinal diameter of specimen in cm	4.0 (1.0-14.0)
Circumferential diameter of specimen in cm	2.5 (0.5-7.0)
Size of specimen in cm^2	9.8 (0.5-70.0)
Circumferential extent of the mucosal defect	
< 75%	268 (71.8)
≥ 75%	105 (28.2)
Histology and depth of invasion	
Intraepithelial neoplasia	131 (35.1)
Mucosa	195 (52.3)
Sub-mucosa < 200 μm	21 (5.6)
Sub-mucosa ≥ 200 μm	26 (7.0)
Procedure time in min	60 (12-240)
En bloc resection	359 (96.2)
Curative resection	321 (86.1)
Type of ESD procedure	
Conventional ESD	33 (8.8)
ESTD	340 (91.2)
Endoscopists	
Experienced	224 (60.1)
Less-experienced	149 (39.9)
Adverse events	
MWT	20 (5.4)
Perforation	6 (1.6)
Stenosis	46 (12.3)

ESD: Endoscopic submucosal dissection; MWT: Mallory-Weiss Tear; ESTD: Endoscopic submucosal tunnel dissection.

the procedure time was 88.5 min (sensitivity, 65.0%; specificity, 70.8%), as calculated by the Youden index method. This result suggested that female patients with procedure times longer than 88.5 min were susceptible to MWTs during esophageal endoscopic resection.

Treatment and clinical courses of MWT patients

Among the 20 patients with MWTs, 6 received endoscopic treatment for the laceration during the ESD procedure. Five patients with slight oozing were treated by APC or endoclips. One patient was treated with 9 endoclips and 1 endoloop to close the laceration, as the mucosal tear was deep and long, with visible vessel and errhysis. The remaining patients did not receive endoscopic hemostasis, and had a benign clinical course, with the exception of one patient (case 7, Table 4) who received a blood transfusion and emergency endoscopy due to delayed bleeding. In this patient,

Table 2 Univariate analysis for risk factors of Mallory-Weiss Tear

	MWT	Non-MWT	*P* value
No. of patients	20	317	
Age in yr, median (range)	65 (47-77)	62 (37-83)	0.065
Sex as male/female	6/14	222/95	[a]0.001
Concomitant disease, *n*			
Atrophic gastritis	0	15	1.000
Hiatal hernia	0	2	1.000
History of distal gastrectomy	0	4	1.000
No. of lesions	20	353	
Location of the center			0.566
Upper	2 (10.0)	19 (5.4)	
Middle	13 (65.0)	239 (67.7)	
Lower	5 (25.0)	95 (26.9)	
Longitudinal diameter in cm, median (range)	5 (2-8)	4 (1-14)	0.125
Circumferential diameter in cm, median (range)	2.75 (1-5)	2.5 (0.5-7)	0.591
Size of specimen in cm^2, median (range)	11.25 (2-32.5)	9.6 (0.5-70)	0.312
Circumferential extent of the mucosal defect			0.085
< 75%	11 (55)	257 (72.8)	
≥ 75%	9 (45)	96 (27.2)	
Depth of invasion			0.377
Intraepithelial neoplasia	4 (20)	127 (36.0)	
Mucosa	13 (65)	182 (51.6)	
Submucosa < 200 μm	1 (5)	20 (5.7)	
Submucosa ≥ 200 μm	2 (10)	24 (6.8)	
Submucosal adhesion, *n*	3 (15)	36 (10.2)	0.452
Procedure time in min, median (range)	90 (28-180)	58 (12-240)	[a]0.008
Type of resection			0.694
Conventional ESD	2 (10)	31 (8.8)	
ESTD	18 (90)	322 (91.2)	
Operators			0.635
Experienced	11(55)	213 (60.3)	
Less-experienced	9 (45)	140 (39.7)	

[a]*P* < 0.05. ESD: Endoscopic submucosal dissection; MWT: Mallory-Weiss Tear; ESTD: Endoscopic submucosal tunnel dissection.

the laceration was not serious at the end of the ESD procedure. However, nausea and vomiting after ESD might have aggravated the laceration, and resulted in hematemesis. In the emergency endoscopy, longitudinal laceration was observed at the esophagastric junction, with visible vessel and blood oozing. After a sprinkle of epinephrine solution (1:10000) and APC, the active bleeding stopped.

Additionally, a severe gas-related adverse event, namely, gastric perforation, occurred in one patient (patient 9, Table 4). The perforation was closed endoscopically by hemoclips and endoloops using a double purse-string suture. As the MWT of the patient was relatively slight without active bleeding, no endoscopic treatment was performed.

In general, most of the patients had a benign clinical course, and no surgery was required for the MWT.

DISCUSSION

This study suggested that the incidence of MWTs during esophageal ESD was 5.4%. Okada *et al*[13] reported a lower incidence of 2.59% (11/424) of MWTs during upper gastrointestinal ESD. However, the authors did not analyze the morbidity separately based on the procedure site.

The incidence of MWTs during screening endoscopy is reported to be 0.08%-

Table 3 Multivariate analysis for Mallory-Weiss Tear during esophageal endoscopic submucosal dissection

Variable	OR	95%CI	*P* value
Age	1.037	0.977-1.101	0.229
Sex, female	5.270	1.940-14.320	[a]0.001
Procedure time, > 88.5 min	3.953	1.497-10.417	[a]0.005

[a]$P < 0.05$.

0.49%[12-15], which is much lower than that of ESD-associated MWTs. We suggest that the longer procedure time, with gas insufflation and the invasive operation, may somehow contribute to the high incidence of MWTs. Endoscopic mucosal resection (EMR) is another treatment method for superficial esophageal neoplasms, especially for small lesions, which have simpler procedure steps with significantly shorter procedure times than ESD. A study[13] identified only one MWT in all 303 patients of esophageal EMR, suggesting a similar incidence to that of diagnostic endoscopy, which indicated that the procedure time was closely associated with the MWT. Accordingly, in our study, univariate and multivariate logistic regression analyses showed that the procedure time was an independent risk factor for an MWT during esophageal ESD.

The development of an endoscopic-associated MWT is closely associated with gas insufflation. An RCT reported that the incidence of MWTs was significantly lower with CO_2 insufflation than with air insufflation (0% *vs* 15.6%) during gastric ESD, because CO_2 can be rapidly absorbed compared to air. However, as the sample size of this study was small, the incidence of MWTs was of low reliability. A Japanese study reported that the incidence of MWTs during gastric ESD is 3.4%, which is lower than that of our study of esophageal ESD. We presume that the direct insight of the stomach is favorable for the supervision of the inflation status of the stomach cavity, and is convenient for gas suction during the ESD procedure.

Predisposing conditions for non-iatrogenic MWTs include chronic alcoholism, the presence of hiatal hernia and atrophic gastritis. Most cases occur after the precipitating factors of fierce vomiting, retching, straining at defecation and coughing[10,17-20]. Scarce reports have suggested associated risk factors for MWTs during screening endoscopy, such as the presence of hiatal hernia, female sex, and a history of distal gastrectomy and old age, while retching or struggling during the procedure remains in dispute[14-16]. In the present study, multivariate logistic regression analysis showed that female sex was an independent risk factor for an MWT. We presumed that the strength of the abdominal muscle in female patients was much weaker than that in male patients. Therefore, the gastric wall could not help to withstand the increasing intragastric pressure caused by the insufflation of gas. This viewpoint could be supported by Kawano *et al*'s[21] study that a low body mass index (< 18.5) is a risk factor for MWT during gastric ESD. Features of the esophageal lesions had no influence on the development of MWTs. Moreover, neither endoscopic findings of atrophic gastritis nor hiatal hernia were found in the 20 MWT patients. This was in accordance with a case-control study showing that hiatal hernia was not associated with MWT[22].

In general, the majority of patients with MWTs have a benign clinical course, and most can be treated conservatively, even without endoscopic intervention[13,15]. Nevertheless, a complicated course, including blood transfusion, rebleeding, surgery and even death, can also occur in 6%-23% of patients when advanced age, active bleeding at initial endoscopy, a lower admission hemoglobin level, clinical symptoms of tarry stool, longer lacerations, and underlying comorbidities are present[13,18,23-26]. In our study, the vast majority of patients did not receive endoscopic treatment, and recovered satisfactorily. We suggest that the placement of a gastrointestinal decompression tube, as well as the use of PPIs and hemostatic drugs after ESD, contributed to hemostasis and mucosal healing. However, only one patient with an MWT who did not receive endoscopic intervention suffered delayed bleeding, and required blood transfusion and emergency endoscopy for hemostasis due to nausea and vomiting after ESD. Six patients with MWTs were treated by endoscopic hemostasis using APC, hemoclips, or hemoclips combined with endoloop during the ESD procedure. Generally, the clinical course and outcome of MWTs during ESD were better than those of classical MWTs. Because post-ESD management was favorable for hemostasis and wound healing, the laceration could be found promptly after the ESD procedure, and could be managed in a timely manner depending on the

Table 4 Clinical characteristics of patients who developed Mallory-Weiss Tear during endoscopic submucosal dissection

Patients	Age in yr	Sex	Location	Circumferential of mucosal defect	Procedure time in min	Expert	Endoscopic treatment of MWT	Invasion depth	Submucosal adhesion
Case 1	72	F	M	3/5	60	Y	-	Tis	N
Case 2	61	M	L	3/4	69	Y	-	Tis	N
Case 3	63	F	M	1/2	28	Y	-	Tis	N
Case 4	47	M	L	1/3	45	Y	-	LGIN	N
Case 5	65	F	M	3/4	90	N	-	HGIN	N
Case 6	64	F	M	4/5	98	Y	-	M2	Y
Case 7	51	F	M	4/5	121	Y	E & APC[1]	M2	N
Case 8	66	F	M	2/3	125	N	-	Tis	N
Case 9	64	F	M	4/5	175	N	-	Tis	N
Case 10	77	M	L	1	180	N	APC	SM, 550 μm	N
Case 11	60	F	M	1/4	45	N	-	LGIN	N
Case 12	67	F	M	2/3	100	N	APC	SM, 100 μm	N
Case 13	67	M	L	1/2	90	N	-	M3	N
Case 14	57	M	M	5/6	155	N	APC	M2	N
Case 15	69	F	M	1/2	57	Y	Clips	HGIN	Y
Case 16	66	F	M	1/2	40	Y	Clips	Tis	Y
Case 17	67	M	L	1	105	Y	Clips and loop	M2	N
Case 18	65	F	M	2/3	89	Y	-	M2	N
Case 19	64	F	U	1	150	Y	-	M2	N
Case 20	67	F	U	2/3	90	N	-	SM, 220 μm	N

[1]Sprinkle of epinephrine solution (1:10000) and argon plasma coagulation treatment in the emergency endoscopy 10 h after endoscopic submucosal dissection. MWT: Mallory-Weiss Tear; Tis: Tumor *in situ*; LGIN: Low-grade intraepithelial neoplasia; HGIN: High-grade intraepithelial neoplasia; APC: Argon plasma coagulation; M2: Lesion limited to laminae mucosa; SM: Lesion infiltration submucosa; F: Female; M: Male; U: Upper esophagus; M: Middle esophagus; L: Lower esophagus.

endoscopic findings. Therefore, it is of great importance to detect the MWT during ESD, and to take optimal endoscopic intervention measures when active bleeding is present.

Endoscopic hemostasis methods for MWT bleeding, including hemoclips, band ligation, epinephrine injection, and electrocoagulation, can achieve a high primary hemostasis rate of more than 90% with a low rebleeding rate and few complications[27-31]. Mechanical methods such as hemoclips and band ligation were superior to epinephrine injection to some extent[28-30].

Although MWT bleeding is mild and can mostly stop spontaneously without endoscopic hemostasis, the spectrum of MWTs is wide and sometimes may result in a fatal condition. Therefore, we recommend an inspection of the esophagastric junction and stomach after the whole procedure of the esophagus to ensure the soundness of the mucosa out of the procedure region. Moreover, inspection during the procedure with gas suction is also proposed when the ESD procedure time is long, especially in female patients. It may help to prevent MWT and avoid further overflow of gas, which may cause further injury. This is essential for the prevention of massive hemorrhage, and even perforation caused by a delayed diagnosis, to avoid unnecessary panic and even surgery or death. In addition, a pressure monitoring system may be useful for the prevention of MWT[32,33].

There are several limitations in the present study. This was a retrospective study from a single center, which may lead to selection bias and a lack of representation. Although the number of MWT cases was large compared with that of other studies, the sample size was still small, as the incidence rate was low. Therefore, further investigations with large sample sizes will be needed.

This is the first study to identify the incidence of MWTs during esophageal ESD, and to analyze the associated risk factors. In conclusion, our study demonstrated that the incidence of MWTs during ESD of superficial esophageal neoplasms is 5.4%. Female sex and a long procedure time (> 88.5 min) are independent risk factors for an MWT. Although all MWT cases recovered satisfactorily, we suggest confirmation of

the mucosa out of the procedure region after ESD for the timely diagnosis and management of MWTs.

ARTICLE HIGHLIGHTS

Research background

Endoscopic submucosal dissection (ESD) has been widely accepted as an effective treatment for superficial esophageal neoplasms when the risk of lymph node metastasis is diagnosed as being very low or negligible. With the development of endoscopic technology and growing emphasis on the diagnosis and endoscopic treatment of early esophageal cancer, the ESD-associated Mallory-Weiss Tear (MWT) is reported case by case.

Research motivation

Adverse events during ESD of superficial esophageal neoplasms, such as perforation and bleeding, have been well-documented. However, MWT during esophageal ESD remains under investigation. The present study was carried out to investigate the incidence and risk factors of MWT during esophageal ESD, since no literature has focused on the risk factors for an MWT during esophageal ESD.

Research objectives

The present study aimed to clarify the incidence of WMTs during esophageal ESD, and to evaluate the associated risk factors.

Research methods

Patients with superficial esophageal neoplasms who received ESD in our institution were retrospectively analyzed. The clinicopathological characteristics of the patients were collected. Patients were divided into a MWT group and non-MWT group based on whether MWT occurred during ESD. The incidence of MWTs was determined, and the risk factors for an MWT were then further explored.

Research results

Twenty patients developed MWTs during ESD (5.4%). Multivariate analysis identified that female sex (OR = 5.36, 95%CI: 1.47-19.50, P = 0.011) and procedure time longer than 88.5 min (OR = 3.953, 95%CI: 1.497-10.417, P = 0.005) were independent risk factors for an MWT during ESD. The cutoff value of the procedure time for an MWT was 88.5 min (sensitivity, 65.0%; specificity, 70.8%).

Research conclusions

The incidence of MWTs during esophageal ESD was much higher than expected. Although most cases have a benign course, fatal conditions may occur. We recommend inspection of the stomach during and after the ESD procedure for timely management in cases of bleeding MWTs or even perforation outside of the procedure region.

Research perspectives

The present study aimed to clarify the incidence of WMTs during esophageal ESD, and to evaluate associated risk factors. In this work, we found that female sex and procedure time were independent risk factors for an MWT during ESD. For patients with such characteristics, clinicians must remain vigilant and perform careful observations after ESD.

REFERENCES

1 **Pimentel-Nunes P**, Dinis-Ribeiro M, Ponchon T, Repici A, Vieth M, De Ceglie A, Amato A, Berr F, Bhandari P, Bialek A, Conio M, Haringsma J, Langner C, Meisner S, Messmann H, Morino M, Neuhaus H, Piessevaux H, Rugge M, Saunders BP, Robaszkiewicz M, Seewald S, Kashin S, Dumonceau JM, Hassan C, Deprez PH. Endoscopic submucosal dissection: European Society of Gastrointestinal Endoscopy (ESGE) Guideline. *Endoscopy* 2015; **47**: 829-854 [PMID: 26317585 DOI: 10.1055/s-0034-1392882]

2 **Choi JY**, Park YS, Jung HY, Ahn JY, Kim MY, Lee JH, Choi KS, Kim DH, Choi KD, Song HJ, Lee GH, Cho KJ, Kim JH. Feasibility of endoscopic resection in superficial esophageal squamous carcinoma. *Gastrointest Endosc* 2011; **73**: 881-889, 889.e1-889.e2 [PMID: 21392755 DOI: 10.1016/j.gie.2010.12.028]

3 **Tsujii Y**, Nishida T, Nishiyama O, Yamamoto K, Kawai N, Yamaguchi S, Yamada T, Yoshio T, Kitamura S, Nakamura T, Nishihara A, Ogiyama H, Nakahara M, Komori M, Kato M, Hayashi Y, Shinzaki S, Iijima H, Michida T, Tsujii M, Takehara T. Clinical outcomes of endoscopic submucosal dissection for superficial esophageal neoplasms: a multicenter retrospective cohort study. *Endoscopy* 2015; **47**: 775-783 [PMID: 25826277 DOI: 10.1055/s-0034-1391844]

4 **Takahashi R**, Yoshio T, Horiuchi Y, Omae M, Ishiyama A, Hirasawa T, Yamamoto Y, Tsuchida T, Fujisaki J. Endoscopic tissue shielding for esophageal perforation caused by endoscopic resection. *Clin J Gastroenterol* 2017; **10**: 214-219 [PMID: 28364314 DOI: 10.1007/s12328-017-0738-z]

5 **Libânio D**, Pimentel-Nunes P, Dinis-Ribeiro M. Complications of endoscopic resection techniques for upper GI tract lesions. *Best Pract Res Clin Gastroenterol* 2016; **30**: 735-748 [PMID: 27931633 DOI: 10.1016/j.bpg.2016.09.010]

6 **Mallory GK**, Weiss S. Hemorrhages from lacerations of the cardiac orifice of the stomach due to vomiting. *Am J Med Sci* 1929; **178**: 506-514 [DOI: 10.1097/00000441-192910000-00005]
7 **Lecleire S**, Di Fiore F, Merle V, Hervé S, Duhamel C, Rudelli A, Nousbaum JB, Amouretti M, Dupas JL, Gouerou H, Czernichow P, Lerebours E. Acute upper gastrointestinal bleeding in patients with liver cirrhosis and in noncirrhotic patients: epidemiology and predictive factors of mortality in a prospective multicenter population-based study. *J Clin Gastroenterol* 2005; **39**: 321-327 [PMID: 15758627 DOI: 10.1097/01.mcg.0000155133.50562.c9]
8 **Halland M**, Young M, Fitzgerald MN, Inder K, Duggan JM, Duggan A. Characteristics and outcomes of upper gastrointestinal hemorrhage in a tertiary referral hospital. *Dig Dis Sci* 2010; **55**: 3430-3435 [PMID: 20407826 DOI: 10.1007/s10620-010-1223-4]
9 **Saylor JL**, Tedesco FJ. Mallory-Weiss syndrome in perspective. *Am J Dig Dis* 1975; **20**: 1131-1134 [PMID: 1200008 DOI: 10.1007/BF01070756]
10 **Knauer CM**. Mallory-Weiss syndrome. Characterization of 75 Mallory-weiss lacerations in 528 patients with upper gastrointestinal hemorrhage. *Gastroenterology* 1976; **71**: 5-8 [PMID: 1084311 DOI: 10.1007/BF02591018]
11 **Watts HD**. Mallory-Weiss syndrome occurring as a complication of endoscopy. *Gastrointest Endosc* 1976; **22**: 171-172 [PMID: 1248717 DOI: 10.1016/S0016-5107(76)73737-4]
12 **Shimoda R**, Iwakiri R, Sakata H, Ogata S, Ootani H, Sakata Y, Fujise T, Yamaguchi K, Mannen K, Arima S, Shiraishi R, Noda T, Ono A, Tsunada S, Fujimoto K. Endoscopic hemostasis with metallic hemoclips for iatrogenic Mallory-Weiss tear caused by endoscopic examination. *Dig Endosc* 2009; **21**: 20-23 [PMID: 19691796 DOI: 10.1111/j.1443-1661.2008.00825.x]
13 **Okada M**, Ishimura N, Shimura S, Mikami H, Okimoto E, Aimi M, Uno G, Oshima N, Yuki T, Ishihara S, Kinoshita Y. Circumferential distribution and location of Mallory-Weiss tears: recent trends. *Endosc Int Open* 2015; **3**: E418-E424 [PMID: 26528495 DOI: 10.1055/s-0034-1392367]
14 **Na S**, Ahn JY, Jung KW, Lee JH, Kim DH, Choi KD, Song HJ, Lee GH, Jung HY, Han S. Risk Factors for an Iatrogenic Mallory-Weiss Tear Requiring Bleeding Control during a Screening Upper Endoscopy. *Gastroenterol Res Pract* 2017; **2017**: 5454791 [PMID: 28348579 DOI: 10.1155/2017/5454791]
15 **Montalvo RD**, Lee M. Retrospective analysis of iatrogenic Mallory-Weiss tears occurring during upper gastrointestinal endoscopy. *Hepatogastroenterology* 1996; **43**: 174-177 [PMID: 8682458 DOI: 10.1016/S0016-5107(05)80149-X]
16 **Penston JG**, Boyd EJ, Wormsley KG. Mallory-Weiss tears occurring during endoscopy: a report of seven cases. *Endoscopy* 1992; **24**: 262-265 [PMID: 1366134 DOI: 10.1055/s-2007-1009122]
17 **Dagradi AE**, Broderick JT, Juler G, Wolinsky S, Stempien SJ. The Mallory-Weiss syndrome and lesion. A study of 30 cases. *Am J Dig Dis* 1966; **11**: 710-721 [PMID: 5946360 DOI: 10.1007/BF02239424]
18 **Kortas DY**, Haas LS, Simpson WG, Nickl NJ, Gates LK. Mallory-Weiss tear: predisposing factors and predictors of a complicated course. *Am J Gastroenterol* 2001; **96**: 2863-2865 [PMID: 11693318 DOI: 10.1111/j.1572-0241.2001.04239.x]
19 **Sugawa C**, Benishek D, Walt AJ. Mallory-Weiss syndrome. A study of 224 patients. *Am J Surg* 1983; **145**: 30-33 [PMID: 6600377 DOI: 10.1016/0002-9610(83)90162-9]
20 **DECKER JP**, ZAMCHECK N, MALLORY GK. Mallory-Weiss syndrome: hemorrhage from gastroesophageal lacerations at the cardiac orifice of the stomach. *N Engl J Med* 1953; **249**: 957-963 [PMID: 13111391 DOI: 10.1056/NEJM195312102492401]
21 **Kawano K**, Nawata Y, Hamada K, Hiroko T, Noriyuki N, Toshihiro N, Kenji T. Examination about the Mallory-Weiss tear as the accident of ESD. *Gastroenterol Endosc* 2012; **54**: 1443-1514
22 **Corral JE**, Keihanian T, Kröner PT, Dauer R, Lukens FJ, Sussman DA. Mallory Weiss syndrome is not associated with hiatal hernia: a matched case-control study. *Scand J Gastroenterol* 2017; **52**: 462-464 [PMID: 28007004 DOI: 10.1080/00365521.2016.1267793]
23 **Kim JW**, Kim HS, Byun JW, Won CS, Jee MG, Park YS, Baik SK, Kwon SO, Lee DK. Predictive factors of recurrent bleeding in Mallory-Weiss syndrome. *Korean J Gastroenterol* 2005; **46**: 447-454 [PMID: 16371719]
24 **Lecleire S**, Antonietti M, Iwanicki-Caron I, Duclos A, Ramirez S, Ben-Soussan E, Hervé S, Ducrotté P. Endoscopic band ligation could decrease recurrent bleeding in Mallory-Weiss syndrome as compared to haemostasis by hemoclips plus epinephrine. *Aliment Pharmacol Ther* 2009; **30**: 399-405 [PMID: 19485979 DOI: 10.1111/j.1365-2036.2009.04051.x]
25 **Fujisawa N**, Inamori M, Sekino Y, Akimoto K, Iida H, Takahata A, Endo H, Hosono K, Sakamoto Y, Akiyama T, Koide T, Tokoro C, Takahashi H, Saito K, Abe Y, Nakamura A, Kubota K, Saito S, Koyama S, Nakajima A. Risk factors for mortality in patients with Mallory-Weiss syndrome. *Hepatogastroenterology* 2011; **58**: 417-420 [PMID: 21661406 DOI: 10.1016/j.dld.2010.10.013]
26 **Ljubičić N**, Budimir I, Pavić T, Bišćanin A, Puljiz Z, Bratanić A, Troskot B, Zekanović D. Mortality in high-risk patients with bleeding Mallory-Weiss syndrome is similar to that of peptic ulcer bleeding. Results of a prospective database study. *Scand J Gastroenterol* 2014; **49**: 458-464 [PMID: 24495010 DOI: 10.3109/00365521.2013.846404]
27 **Cho YS**, Chae HS, Kim HK, Kim JS, Kim BW, Kim SS, Han SW, Choi KY. Endoscopic band ligation and endoscopic hemoclip placement for patients with Mallory-Weiss syndrome and active bleeding. *World J Gastroenterol* 2008; **14**: 2080-2084 [PMID: 18395910 DOI: 10.3748/wjg.14.2080]
28 **Chung IK**, Kim EJ, Hwang KY, Kim IH, Kim HS, Park SH, Lee MH, Kim SJ. Evaluation of endoscopic hemostasis in upper gastrointestinal bleeding related to Mallory-Weiss syndrome. *Endoscopy* 2002; **34**: 474-479 [PMID: 12048631 DOI: 10.1055/s-2002-32000]
29 **Park CH**, Min SW, Sohn YH, Lee WS, Joo YE, Kim HS, Choi SK, Rew JS, Kim SJ. A prospective, randomized trial of endoscopic band ligation vs. epinephrine injection for actively bleeding Mallory-Weiss syndrome. *Gastrointest Endosc* 2004; **60**: 22-27 [PMID: 15229420 DOI: 10.1016/S0016-5107(04)01284-2]
30 **Peng YC**, Tung CF, Chow WK, Chang CS, Chen GH, Hu WH, Yang DY. Efficacy of endoscopic isotonic saline-epinephrine injection for the management of active Mallory-Weiss tears. *J Clin Gastroenterol* 2001; **32**: 119-122 [PMID: 11205645 DOI: 10.1097/00004836-200102000-00005]
31 **Papp JP**. Electrocoagulation of actively bleeding Mallory-Weiss tears. *Gastrointest Endosc* 1980; **26**: 128-130 [PMID: 7450460 DOI: 10.1016/S0016-5107(80)73301-1]
32 **Takada J**, Araki H, Onogi F, Nakanishi T, Kubota M, Ibuka T, Shimizu M, Moriwaki H. Safety and efficacy of carbon dioxide insufflation during gastric endoscopic submucosal dissection. *World J Gastroenterol* 2015; **21**: 8195-8202 [PMID: 26185394 DOI: 10.3748/wjg.v21.i26.8195]
33 **Nakajima K**, Moon JH, Tsutsui S, Miyazaki Y, Yamasaki M, Yamada T, Kato M, Yasuda K, Sumiyama

K, Yahagi N, Saida Y, Kondo H, Nishida T, Mori M, Doki Y. Esophageal submucosal dissection under steady pressure automatically controlled endoscopy (SPACE): a randomized preclinical trial. *Endoscopy* 2012; **44**: 1139-1148 [PMID: 22932809 DOI: 10.1055/s-0032-1310093]

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ORIGINAL ARTICLE

Clinical Trials Study

Overlay of a sponge soaked with ropivacaine and multisite infiltration analgesia result in faster recovery after laparoscopic hepatectomy

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Abstract

BACKGROUND

Compared with traditional open surgery, laparoscopic surgery is preferred due to the advantages of less trauma, less pain, and faster recovery. Nevertheless, many patients still suffer from postoperative pain resulting from the surgical incision and associated tissue injury. Many researchers have reported methods to improve postoperative pain control, but there is not a simple and effective method that can be clinically adopted in a widespread manner. We designed this study to prove the hypothesis that application of ropivacaine in the port site and operative site in patients is an effective and convenient method which can decrease postoperative pain and accelerate recovery.

AIM

To evaluate the effects of ropivacaine on pain control after laparoscopic hepatectomy and its contribution to patient recovery.

METHODS

From May 2017 to November 2018, 146 patients undergoing laparoscopic hepatectomy were randomized to receive infiltration of either 7.5 mg/mL ropivacaine around the trocar insertions, incision, and cutting surface of the liver (with a gelatin sponge soaked with ropivacaine) at the end of surgery (ropivacaine group), or normal saline (5 mL) at the same sites at the end of surgery (control group). The degree of pain, nausea, vomiting, heart rate (HR), and blood pressure were collected. The length of postoperative hospitalization, complications, and the levels of stress hormones were also compared between the two groups.

RESULTS

Compared with the control group, the ropivacaine group showed reduced

study inclusion.

Conflict-of-interest statement: We declare that we have no conflicts of interest.

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postoperative pain at rest within 12 h ($P < 0.05$), and pain on movement was reduced within 48 h. The levels of epinephrine, norepinephrine, and cortisol at 24 and 48 h, HR, blood pressure, and cumulative sufentanil consumption in the ropivacaine group were significantly lower than those in the control group ($P < 0.05$). In the ropivacaine group, hospitalization after operation was shorter, but the difference was not statistically significant. There were no significant differences in postoperative nausea, vomiting, or other complications, including hydrothorax, ascites, peritonitis, flatulence, and venous thrombus ($P > 0.05$), although fewer patients in the ropivacaine group experienced these situations.

CONCLUSION
Infiltration with ropivacaine in the abdominal wound and covering the cutting surface of the liver with a gelatin sponge soaked with ropivacaine significantly reduce postoperative pain and the consumption of sufentanil.

Key words: Postoperative pain; Local anesthetics; Ropivacaine; Laparoscopic hepatectomy; Gelatin sponge

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Core tip: This study confirmed the efficacy of ropivacaine in pain control after laparoscopic hepatectomy and its contribution to fast track recovery surgery. Ropivacaine not only infiltrated the subcutaneous and deep muscle fasciae and peritoneum but also covered the liver cutting surface in a soaked gelatin sponge to relieve the pain caused by capsule injury. We examined the efficacy using not only visual analog scale, but also blood biochemistry and other standards.

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INTRODUCTION

For hepatectomy, laparoscopic surgery is preferred in the clinic with the advantages of less trauma, less pain, faster recovery, and a shorter hospital stay than traditional open surgery[1]. However, many patients still suffer from postoperative pain resulting from the surgical incision and associated tissue injury, which lengthens the bedridden time and increases the risk of complications. Therefore, optimal and reliable analgesia for postoperative pain control is very important. Thus, many researchers have reported methods to improve postoperative pain control, accelerate recovery, and decrease hospital stays, such as local anesthesia at the trocar site[2], decreased pneumoperitoneum[3], and intraperitoneal nebulization of anesthetic[4]. Therefore, many methods have been developed and have proven useful, but there is not a simple and effective method that can be clinically adopted in a widespread manner. The focal point of the controversy regarding local anesthesia after an operation centers around the following questions: What the anesthetic is, what the concentration is, and where it should be used. Most previous trials have estimated the degree of analgesia using a visual analog scale (VAS), which is rather subjective. Therefore, we designed this case-control study to examine the efficacy of multisite infiltrative analgesia in trocar sites and sponges soaked with ropivacaine after laparoscopic hepatectomy using VAS, blood biochemistry, and other standards.

To evaluate the effects of ropivacaine on pain control after laparoscopic hepatectomy, we designed a randomized, double-blind, placebo-controlled clinical trial to find an effective and convenient method to decrease postoperative pain and accelerate recovery.

MATERIALS AND METHODS

Subject population

The study was approved by the Ethics Committee of Qilu Hospital of Shandong University (No. 2017052). From May 2017 to November 2018, 146 patients (age range: 20-80 years; weight: 50-90 kg) undergoing laparoscopic hepatectomy performed by our team at the Department of Surgery, Qilu Hospital of Shandong University (Jinan, China) were included in this study. All patients signed an informed consent form before the operation. Patients were excluded if they had acute cholecystitis, serious chronic pain disease, history of alcohol abuse, tranquilizer treatment before surgery, cognitive impairment, or history of allergy to any of the drugs used in the study.

Before the start of the study, random sequence was generated with the Statistical Package for Social Sciences (SPSS) 20.0 (SPSS Inc., Chicago, IL, United States). The patients were randomized into two groups with the table of random numbers on the day of surgery. A group of patients received ropivacaine infiltration around the trocar insertion and abdominal wounds for specimen extraction, and another group of patients received the same volume of normal saline; at the end of the operation, uniform disposal was similar across groups.

Anesthesia methods and surgery

All patients received standard general anesthesia administered by the same anesthetist not involved in the study. Anesthesia was induced with propofol, midazolam, rocuronium, and sufentanil. General anesthesia was maintained with 2% sevoflurane.

Twenty milliliter solutions containing either 7.5 mg/mL ropivacaine for the ropivacaine group or normal saline for the control group were prepared and provided by an identical anesthetist, and the surgeons were not informed of the contents of the solution or of the patient allocation. Before trocar withdrawal, a gelatin sponge was soaked with 10 mL of the prepared solution and used to cover the cutting surface of the liver. At the end of the surgery, subcutaneous tissue and deep muscle around the trocar incision and abdominal wound for specimen extraction were infiltrated with 10 mL of the solution with a needle introduced through the skin. Finally, one drainage tube was routinely used for the patient, and the tissue surrounding the tube was also infiltrated with the solution. All patients were given a patient-controlled analgesic (PCA) device containing 100 mL of 1 μg/mL sufentanil that was delivered at a rate of 2 μg/h to the venous catheter. When the patient felt pain, they pushed a button on the device, and 0.5 mL of additional sufentanil was delivered. The gelatin sponge used in the two groups were the same (Xiangen Medical Technology Development Co., Ltd., Jiangxi, China).

Data collection

During the preoperative communication, all patients were taught how to describe their postoperative pain level using a VAS ranging from 0 (no pain) to 10 (maximal pain). Pain was assessed with the VAS at rest and on movement including coughing and leaning to one side. Pain scores, nausea, vomiting, mean blood pressure, and heart rate (HR) were recorded before the operation and at 0 h, 6 h, 12 h, 24 h, and 48 h after the operation. The cumulative consumption of sufentanil by the PCA device was recorded at 6, 12, 24, and 36 h. If the patients experienced nausea or vomiting, metoclopramide was given. Blood was sampled for detection of levels of epinephrine, norepinephrine, and cortisol at 0 h, 24 h, and 48 h after the operation. Quantitative changes in the levels of catecholamines, including adrenaline, noradrenaline, and dopamine, were analyzed. The surgical stress hormones (epinephrine, norepinephrine, and cortisol) were detected using commercial enzyme-linked immunosorbent assay kits (Cusabio Biotech Co., Ltd., Wuhan, China).

Statistical analysis

A total of 146 patients were included in the study from May 2017 to November 2018. Finally, 69 patients in the ropivacaine group and 67 patients in the control group were included in the statistical analysis.

Continuous data (age, weight, duration of surgery, trocar number, incision length, VAS, and level of hormone) are presented as the mean ± SD. These data were analyzed by *t*-tests. The *P*-value and 95% confidence interval (CI) were calculated. Categorical data [sex, American society of anesthesiologists (ASA) grade, and operation type] are presented as the number of patients and were analyzed by chi-square tests. All data were checked for normal distribution. Data analysis was accomplished with SPSS 20.0 (SPSS Inc., Chicago, IL, United States). $P < 0.05$ was considered statistically significant.

RESULTS

In the end, 139 patients successfully received the surgery, including laparoscopic hepatectomy and the use of the prearranged solution. Four patients and three patients were converted to open laparotomy due to operational difficulties and uncontrolled bleeding during the operation, respectively. Subsequently, three additional patients were excluded from the study: One in the ropivacaine group (because of a second operation for bile leakage) and two in the control group (due to a transfer to the intensive care unit for postoperative dyspnea and a second operation for postoperative hemorrhage). For the statistical analysis, 69 patients in the ropivacaine group and 67 patients in the control group were included.

The demographic data, ASA grade, and details of the surgery, including the surgery type, duration, trocar number, and the length of incision for specimen extraction, were similar between the two groups (Table 1). Postoperative pain scores were significantly lower at rest in the ropivacaine group than in the control group, at 0 h, 6 h, and 12 h and significantly lower on movement at 0 h, 6 h, 12 h, and 24 h ($P < 0.05$). Mean arterial pressure (MAP) was significantly higher in the control group than in the ropivacaine group at 6 h ($P = 0.002$) and 12 h ($P = 0.032$) (Figure 1 and Table 2).

The magnitude of the changes in epinephrine, norepinephrine and cortisol levels above the normal levels was significantly different between the two groups at 24 h and 48 h after surgery (Figure 2A-C, Table 3). Cumulative sufentanil consumption was significantly different between the two groups at 6 h, 12 h, 24 h, and 36 h ($P < 0.05$ for all comparisons, Figure 2D and Table 2).

Postoperative hospitalization was shorter in the ropivacaine group (7.24 ± 2.50) than in the control group (7.70 ± 2.95), but there was no significant difference ($P = 0.324$, Table 4). There were similar findings between the two groups regarding vomiting (55.7% and 60.0% for the ropivacaine group and the control group, respectively), and the difference between the two groups for nausea (12.9% and 18.6% for the ropivacaine group and the control group, respectively) was not statistically significant (Table 4). Other complications, including hydrothorax, ascites, peritonitis, flatulence, venous thrombus, and incision infection, in the two groups were not significantly different ($P = 0.339$) (Table 4).

DISCUSSION

Compared with open surgery, the laparoscopic technique has largely reduced surgical trauma and hospitalization; however, postoperative pain is still an important problem for physicians and patients[5]. Postoperative pain is a phenomenon in which different variables must be taken into account[6]. Surgical incision is the main origin of the pain stimuli, which is influenced by the length of the incision, the number of trocars, and trauma occurring when removing the specimen. Another important factor is pneumoperitoneum created with CO_2, which causes peritoneal stretch, diaphragmatic stretch and chemical irritation, and the attendant carbonic acid formation. Additional causes of pain may involve increased inflammatory mediators after tissue injury, increased lactate concentrations and low pH in the skin and muscle wounds, and central neuronal sensitization[7]. To date, not all of these components are known to be involved, and therapeutic approaches still need to be improved.

Ropivacaine is a long-acting amino amide local anesthetic drug. Ropivacaine blocks the generation and conduction of nerve impulses, presumably by increasing the threshold for electrical excitation in the nerve, by slowing the propagation of the nerve impulse, and by reducing the rate of rise of the action potential[8]. Ropivacaine is a well-tolerated regional anesthetic effective for surgical anesthesia and postoperative relief. The nerve block effect is related to the drug concentration. When the concentration of ropivacaine is 0.2%, the sensory nerve block effect is better, but there is almost no motor nerve block effect; when the concentration is 0.75%, the nerve block effect is better. This "separation block" is the characteristic effect of ropivacaine. Thus, ropivacaine, with its efficacy, low propensity for motor block, and reduced potential for central nervous system (CNS) toxicity and cardiotoxicity, appears to be an important option for regional anesthesia and the management of postoperative pain[9]. In this study, adverse effects of ropivacaine for local anesthesia, such as allergic reactions, local tissue toxicity, cardiovascular toxicity, central nervous system toxicity, and systemic toxicity, were not observed.

Measures that have been reported to assist in the treatment of postoperative pain include inducing local anesthesia, decreasing pneumoperitoneum pressure[3], and administering non-steroidal anti-inflammatory drugs (NSAIDs) and opioids[10]. We found several trials that assessed the effects of local anesthesia in intraperitoneal,

Table 1 Demographic characteristics of the 140 patients studied (mean ± SD)

Characteristic	Ropivacaine group	Control group	t/χ^2	*P*-value
Age (yr)	52.87 ± 12.463	50.31 ± 13.984	1.126	0.2621
Sex				
Male/female	38/31	38/29	38/29	0.8469
Weight (kg)	67.88 ± 11.634	65.75 ± 11.992	1.051	0.2951
ASA grade				
I/II/III	8/44/17	12/42/13	1.351	0.5090
Duration of surgery (min)	156.16 ± 82.748	152.75 ± 87.325	0.234	0.8153
Trocar number	4.58 ± 0.715	4.73 ± 0.770	-1.190	-1.190
Incision length (cm)	5.25 ± 1.035	5.45 ± 0.784	-1.276	0.2040
Operation type				
Lap right hepatectomy	5	6	5.230	0.1557
Lap left hepatectomy	21	12		
Lap caudate hepatectomy	1	5		
Lap irregular hepatectomy	42	44		

ASA: American Society of Anesthesiologists; Lap: Laparoscopic.

subdiaphragmatic, and trocar sites, which significantly decreased postoperative pain scores[11,12]. Postoperative pain mainly comes from wounds of the abdominal muscle and peritoneum. Beyond those causes, injury of Glisson's capsule can also influence pain[13,14]. Therefore, in our study, ropivacaine not only infiltrated the subcutaneous and deep muscle fasciae and peritoneum but also covered the liver cutting surface in a soaked gelatin sponge to relieve the pain caused by capsule injury.

This study has shown the analgesic efficacy of local infiltrative and soaked sponge cover with ropivacaine after laparoscopic hepatectomy, suggesting the method as a reliable strategy for analgesia after operation. Based on the pain score at rest and the significantly lower movement seen in the ropivacaine group than in the control group, ropivacaine was considered to have a clear analgesic effect that lasted approximately 24 h.

Surgical stress could cause changes in the neuroendocrine, metabolic, immune, and hematological systems[15]. Surgical stress is mainly caused by injury during the operation, including somatic and visceral trauma. Surgical stress causes a series of hormonal changes; in the study, we chose catecholamines (epinephrine and norepinephrine) and cortisol as a reference to estimate the degree of surgery stress[16]. The levels of epinephrine, norepinephrine, and cortisol were significantly reduced in the first 48 h after surgery in the ropivacaine group compared to the control group, which means that the ropivacaine method produced clear effects. The use of ropivacaine significantly reduced surgical stress.

Pain relief plays a leading role in enhanced recovery after surgery (ERAS), which will bring many benefits[17]. Theoretically, exercising the lower limbs will promote blood circulation, which can reduce the formation of venous thrombosis[18]. Early ambulation increases lung ventilation, which promotes the exclusion of tracheal secretions and prevents pulmonary complications[19]. Early ambulation after surgery can also promote gastrointestinal peristalsis, reduce abdominal distention, improve appetite, and prevent constipation. However, in the present study, the complications described above showed no significant difference between the groups. One reason may be that compared with traditional open surgery, the laparoscopic technique clearly reduced pain, so the complications between the two groups were not sufficiently distinct. Another potential reason is that the incidence of thrombus of the lower extremity veins and incision infection is low in laparoscopic hepatectomy, so the differences were not significant with a limited sample size.

Although the results support the notion that multisite local infiltration and the sponge soaked with ropivacaine significantly decreased postoperative pain, there are also deficiencies in this study. First, we have not validated the analgesic effects of ropivacaine at different concentrations. More research is needed to determine the best concentration and dose used for local anesthesia after laparoscopic hepatectomy. Second, this study did not determine whether the analgesic effect of ropivacaine was effective for all patients or whether sex, age, weight, disease conditions, and other factors influence the analgesic effect of ropivacaine. Third, the adverse reactions of ropivacaine, such as wound infection and muscle damage, were not significantly

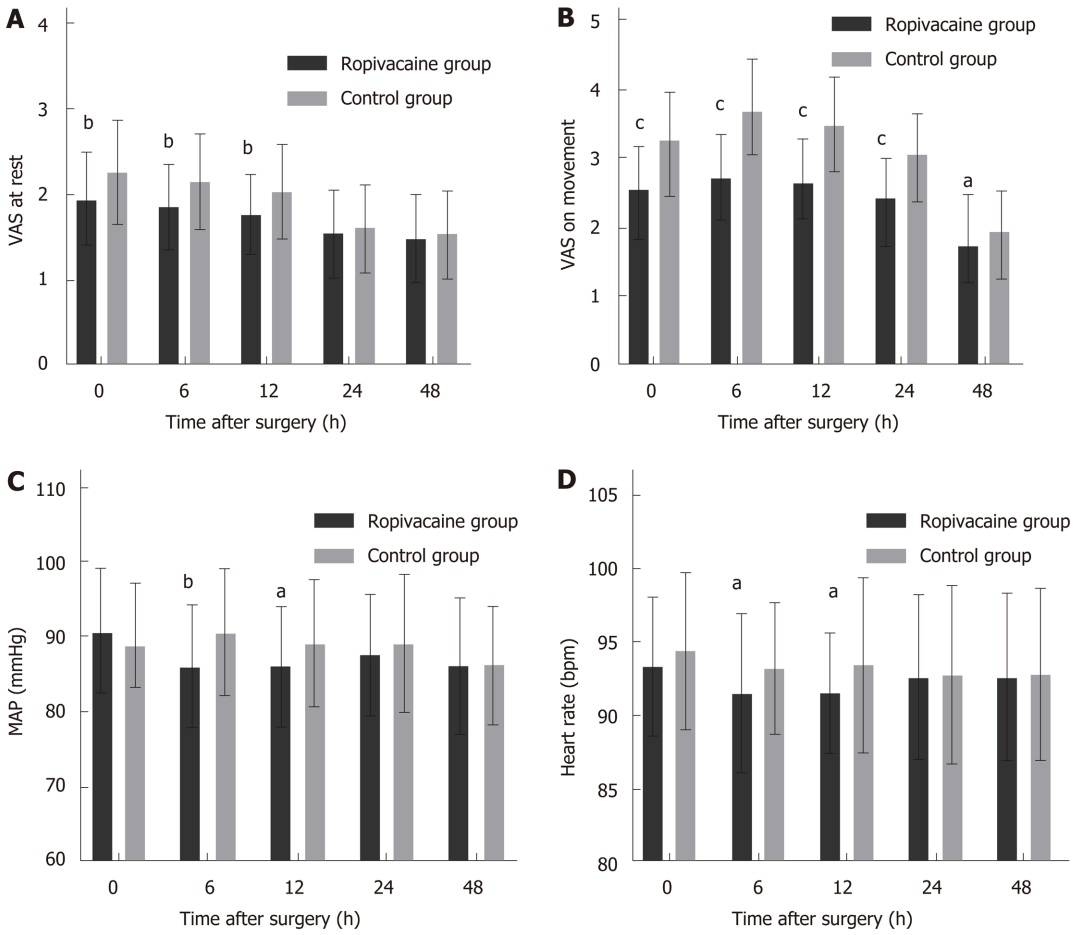

Figure 1 Visual analog scale scores for pain at rest and pain on movement, mean arterial pressure, and heart rate during the first 48 h after surgery. A: Visual analog scale score at rest; B: Visual analog scale score on movement; C: Mean arterial pressure; D: Heart rate. $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.0001$. VAS: Visual analog scale; MAP: Mean arterial pressure; HR: Heart rate.

different in this study, which may have been because the sample was not large enough or the ropivacaine concentration was too low. Moreover, the incidence of nausea, vomiting, and abdominal distension caused mainly by general anesthesia and pneumoperitoneum is not obviously reduced by the use of ropivacaine-soaked sponges. To overcome these deficiencies, more research is needed.

Taken together, local anesthesia with ropivacaine is a simple and inexpensive way to achieve analgesic effects after laparoscopic hepatectomy (Figure 3). With sponges soaked with ropivacaine covering the cutting surface of the liver, the concentration of ropivacaine can be maintained for a longer time with a lower incidence of abdominal infection than seen from the intraperitoneal administration of ropivacaine. Ropivacaine infiltration anesthesia reduces postoperative somatic pain, while sponges soaked with ropivacaine can reduce visceral pain and last for a long time, thus avoiding the administration of high doses of opioid analgesic drugs. In addition to analgesia, ropivacaine can also shorten hospitalization and the time spent in bed, potentially leading to a faster recovery. Considering the simplicity, safety, and efficacy of multisite local anesthesia and the sponge covering of the liver with ropivacaine after laparoscopic hepatectomy, this method is very worthy of application and promotion. In the future, the method could be applied to more areas such as gastrectomy and rectal resection with further study.

Table 2 Visual analog scale scores for pain at rest and pain on movement, mean arterial pressure, and heart rate during the first 48 h after surgery

Characteristic	0 h	6 h	12 h	24 h	48 h[1]
VAS at rest					
Ropivacaine group	1.94 ± 0.539	1.86 ± 0.493	1.77 ± 0.458	1.54 ± 0.502	1.49 ± 0.504
Control group	1.49 ± 0.504	2.15 ± 0.557	2.03 ± 0.550	1.63 ± 0.487	1.54 ± 0.502
t	-3.155	-3.362	-3.019	-1.068	-0.516
P-value	0.0020	0.0014	0.0030	0.2876	0.6064
95%CI	[-0.523, -0.136]	[-0.478, -0.126]	[-0.461, -0.117]	[-0.258, 0.074]	[-0.233, 0.104]
VAS on movement					
Ropivacaine group	2.55 ± 0.631	2.74 ± 0.610	2.65 ± 0.614	2.42 ± 0.579	1.75 ± 0.604
Control group	3.27 ± 0.687	3.70 ± 0.739	3.51 ± 0.660	3.06 ± 0.574	1.97 ± 0.521
t	-6.349	-8.691	-7.991	-6.021	-1.903
P-value	<0.0001	<0.0001	<0.0001	<0.0001	0.0271
95%CI	[-0.942, -0.494]	[-1.192, -0.733]	[-1.071, -0.639]	[-0.835, -0.444]	[-0.408, -0.025]
MAP (mmHg)					
Ropivacaine group	90.81 ± 8.432	86.00 ± 8.070	86.07 ± 7.930	87.61 ± 8.037	86.17 ± 9.160
Control group	88.78 ± 8.348	90.54 ± 8.412	89.15 ± 8.504	89.24 ± 9.160	86.28 ± 7.792
t	1.414	-3.210	-2.183	-1.104	-0.075
P-value	0.1596	0.0017	0.0308	0.2716	0.9402
95%CI	[-0.811, 4.882]	[-7.333, -1.742]	[-5.864, -0.289]	[-4.550, 1.290]	[-2.998, 2.779]
HR (bpm)					
Ropivacaine group	93.36 ± 4.706	91.57 ± 5.377	91.58 ± 4.120	92.60 ± 5.592	92.63 ± 5.696
Control group	94.42 ± 5.326	93.25 ± 4.427	93.46 ± 5.925	92.81 ± 6.079	92.83 ± 5.828
t	-1.231	-1.991	-2.155	-0.214	-0.202
P-value	0.2204	0.0486	0.0331	0.8308	0.8406
95%CI	[-2.768, 0.644]	[-3.348, -0.011]	[-3.617, -0.146]	[-2.197, 1.768]	[-2.154, 1.756]
Cumulative sufentanil (μg)					
Ropivacaine group	0	13.66 ± 2.437	27.91 ± 4.176	55.60 ± 6.117	81.66 ± 7.729
Control group	0	15.43 ± 3.273	30.93 ± 6.414	61.45 ± 5.405	88.89 ± 7.937
t		-3.579	-3.242	-5.897	-5.386
P-value		0.0005	0.0016	<0.0001	<0.0001
95%CI		[-2.744, -0.791]	[-4.863, -1.174]	[-7.803, -3.884]	[-9.891, -4.578]

[1]The time point with cumulative sufentanil is 36 h. CI: Confidence interval; VAS: Visual analog scale; MAP: Mean arterial pressure; HR: Heart rate.

Table 3 The levels of stress hormones, including epinephrine, norepinephrine, and cortisol, and cumulative sufentanil consumption during the first 48 h after the operation

Stress hormone	0 h	24 h	48 h
Epinephrine			
Ropivacaine group	58.05 ± 19.614	76.48 ± 23.884	81.58 ± 24.529
Control group	57.60 ± 17.608	94.29 ± 28.439	100.89 ± 27.413
t	0.141	-3.959	-4.333
P-value	0.8879	0.0001	<0.0001
95%CI	[-5.876, 6.780]	[-26.707, -8.913]	[-28.131, -10.498]
Norepinephrine			
Ropivacaine group	220.57 ± 27.623	252.01 ± 29.539	270.68 ± 30.792
Control group	222.52 ± 27.907	296.90 ± 32.601	322.12 ± 34.942
t	-0.409	-8.421	-9.116
P-value	0.6831	<0.0001	<0.0001
95%CI	[-11.367, 7.470]	[-55.441, -34.351]	[-62.602, -40.280]
Cortisol			
Ropivacaine group	325.82 ± 29.790	365.06 ± 35.820	417.79 ± 36.971
Control group	320.74 ± 34.618	393.82 ± 37.302	438.14 ± 40.814
t	0.918	-4.586	-3.048
P-value	0.3600	<0.0001	0.0028
95%CI	[-5.862, 16.025]	[-41.158, -16.356]	[-33.544, -7.145]

CI: Confidence interval.

Table 4 Postoperative hospitalization and complications

Characteristic	Ropivacaine group	Control group	t/χ^2	*P*-value
Hospitalization after operation (d)	7.28 ± 2.502	7.76 ± 2.990	-1.029	0.3055
Postoperative nausea and vomiting				
Neither nausea nor vomiting	22	15	1.819	0.4027
Nausea without vomiting	39	41		
Nausea with vomiting	8	11		
Complication (+/-)				
Hydrothorax	25/44	27/40	0.238	0.6256
Ascites	10/59	8/59	0.193	0.6606
Peritonitis	1/68	0/67	0.978	0.3226
Flatulence	32/37	39/28	1.907	0.1673
Venous thrombus	3/66	5/62	0.596	0.4402
Incision infection	2/67	1/66	0.312	0.5768

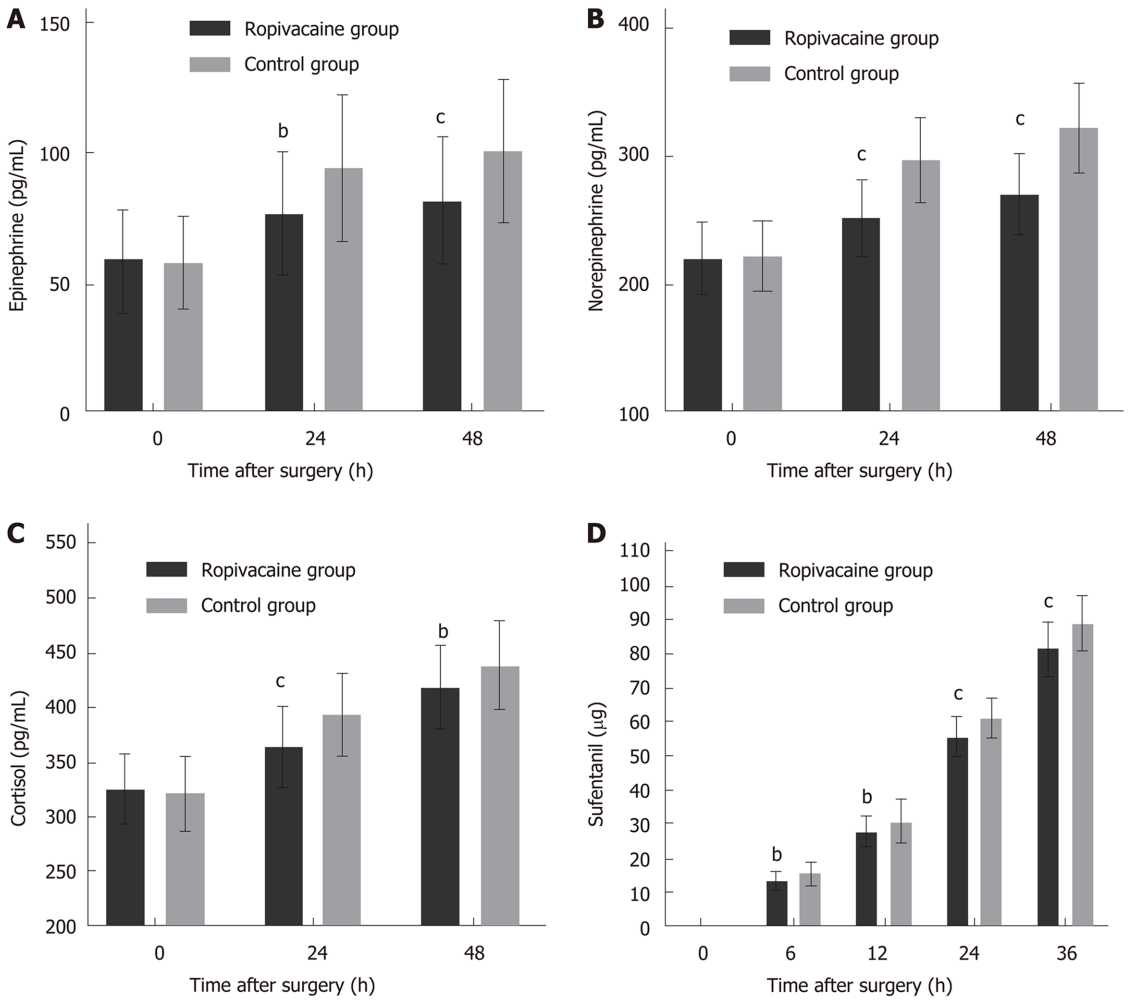

Figure 2 The levels of stress hormones, including epinephrine, norepinephrine, and cortisol, and cumulative sufentanil consumption during the first 48 h after the operation. A: Epinephrine; B: Norepinephrine; C: Cortisol; D: Cumulative sufentanil consumption. $^{b}P < 0.01$, $^{c}P < 0.0001$.

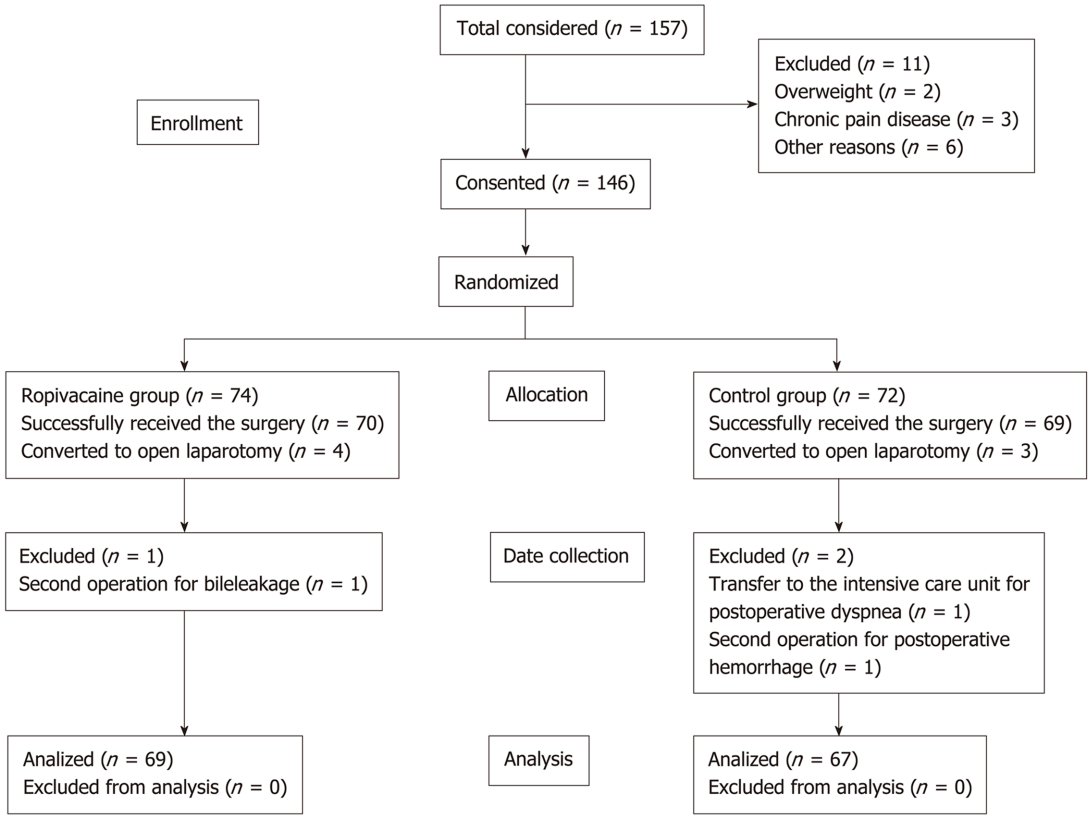

Figure 3 The process of research, including the enrollment and allocation of patients as well as the collection and analysis of data.

ARTICLE HIGHLIGHTS

Research background

The postoperative pain caused by laparoscopic hepatectomy delays patients' recovery, although the pain has reduced a lot compared with laparotomy. Although many methods have been reported for pain relief including intravenous analgesia, epidural analgesia and so on, these methods have their limitations and contraindications. Local wound infiltration is a simple and safe method that can effectively relieve the pain after surgery. The current study was designed to evaluate the effects of ropivacaine on pain control after laparoscopic hepatectomy and to examine whether this local anesthetic technique accelerates patient recovery, thus contributing to the idea of fast track recovery surgery.

Research motivation

Many methods have been reported to improve postoperative pain control, but there is not a simple and effective method that can be clinically adopted in a widespread manner. We designed this study to find an effective and convenient method which can be can be clinically adopted to decrease postoperative pain and accelerate recovery.

Research objectives

The aim of the study was to assess the effectiveness of ropivacaine injections in the port site as well as in the operative site in patients undergoing a hepatectomy.

Research methods

Before the start of the study, random sequence was generated to make sure that the allocation was completely random. For allocation concealment, envelope method was adopted. Continuous data were analyzed by *t*-tests. The *P*-value and 95% confidence interval were calculated. Categorical data were analyzed by chi-square tests. All data were checked for normal distribution. Double blindness was ensured, and the researcher in charge of allocation and anesthetic preparation did not participate in the data collection and analysis.

Research results

Infiltration with ropivacaine in the abdominal wound and covering the cutting surface of the liver with a gelatin sponge soaked with ropivacaine could provide effective analgesia after laparoscopic hepatectomy, with a lower visual analog scale (VAS) score and sufentanil consumption, accelerated postoperative recovery, and reduced stress response. These results suggest that this method is a simple, convenient, and effective analgesic method that can provide postoperative analgesia and short-term benefits after surgery. There are still problems need to be

solved: The best concentration and dose used for local anesthesia need more research to determine, and more effective anesthetics and better method of application need to be found.

Research conclusions

This study provides evidence supporting that infiltration with ropivacaine in the abdominal wound and covering the cutting surface of the liver with a gelatin sponge soaked with ropivacaine after laparoscopic hepatectomy can improve postoperative pain relief, reduce surgical stress response, and accelerate postoperative recovery. This method is very worthy of application and promotion for its simplicity, safety and efficacy.

Research perspectives

In this study, the effect of pain relief of ropivacaine remained no more than 24 h to 48 h, so anesthetics with a more lasting effect need to be found. Furthermore, more research is needed to determine the best concentration and dose used for local anesthesia. Methods of anesthetics application are variable, so researchers could try to find more effective and convenient techniques for pain relief.

REFERENCES

1 **Lim KH**, Chung BS, Kim JY, Kim SS. Laparoscopic surgery in abdominal trauma: A single center review of a 7-year experience. *World J Emerg Surg* 2015; **10**: 16 [PMID: 26056529 DOI: 10.1186/s13017-015-0007-8]

2 **Das NT**, Deshpande C. Effects of Intraperitoneal Local Anaesthetics Bupivacaine and Ropivacaine versus Placebo on Postoperative Pain after Laparoscopic Cholecystectomy: A Randomised Double Blind Study. *J Clin Diagn Res* 2017; **11**: UC08-UC12 [PMID: 28893013 DOI: 10.7860/JCDR/2017/26162.10188]

3 **Singla S**, Mittal G, Raghav, Mittal RK. Pain management after laparoscopic cholecystectomy-a randomized prospective trial of low pressure and standard pressure pneumoperitoneum. *J Clin Diagn Res* 2014; **8**: 92-94 [PMID: 24701492 DOI: 10.7860/JCDR/2014/7782.4017]

4 **Allegri M**, Ornaghi M, Ferland CE, Bugada D, Meghani Y, Calcinati S, De Gregori M, Lovisari F, Radhakrishnan K, Cusato M, Scalia Catenacci S, Somaini M, Fanelli G, Ingelmo P. Peritoneal Nebulization of Ropivacaine during Laparoscopic Cholecystectomy: Dose Finding and Pharmacokinetic Study. *Pain Res Manag* 2017; **2017**: 4260702 [PMID: 28316464 DOI: 10.1155/2017/4260702]

5 **Kasai M**, Cipriani F, Gayet B, Aldrighetti L, Ratti F, Sarmiento JM, Scatton O, Kim KH, Dagher I, Topal B, Primrose J, Nomi T, Fuks D, Abu Hilal M. Laparoscopic versus open major hepatectomy: A systematic review and meta-analysis of individual patient data. *Surgery* 2018; **163**: 985-995 [PMID: 29555197 DOI: 10.1016/j.surg.2018.01.020]

6 **Brennan TJ**. Pathophysiology of postoperative pain. *Pain* 2011; **152**: S33-S40 [PMID: 21232860 DOI: 10.1016/j.pain.2010.11.005]

7 **Carvalho B**, Clark DJ, Angst MS. Local and systemic release of cytokines, nerve growth factor, prostaglandin E2, and substance P in incisional wounds and serum following cesarean delivery. *J Pain* 2008; **9**: 650-657 [PMID: 18394968 DOI: 10.1016/j.jpain.2008.02.004]

8 **Toda S**, Sakai A, Ikeda Y, Sakamoto A, Suzuki H. A local anesthetic, ropivacaine, suppresses activated microglia via a nerve growth factor-dependent mechanism and astrocytes via a nerve growth factor-independent mechanism in neuropathic pain. *Mol Pain* 2011; **7**: 2 [PMID: 21211063 DOI: 10.1186/1744-8069-7-2]

9 **Kuthiala G**, Chaudhary G. Ropivacaine: A review of its pharmacology and clinical use. *Indian J Anaesth* 2011; **55**: 104-110 [PMID: 21712863 DOI: 10.4103/0019-5049.79875]

10 **Shibasaki S**, Kawamura H, Homma S, Yosida T, Takahashi S, Takahashi M, Takahashi N, Taketomi A. A comparison between fentanyl plus celecoxib therapy and epidural anesthesia for postoperative pain management following laparoscopic gastrectomy. *Surg Today* 2016; **46**: 1209-1216 [PMID: 26695406 DOI: 10.1007/s00595-015-1290-4]

11 **Sun JX**, Bai KY, Liu YF, Du G, Fu ZH, Zhang H, Yang JH, Wang B, Wang XY, Jin B. Effect of local wound infiltration with ropivacaine on postoperative pain relief and stress response reduction after open hepatectomy. *World J Gastroenterol* 2017; **23**: 6733-6740 [PMID: 29085218 DOI: 10.3748/wjg.v23.i36.6733]

12 **Bisgaard T**, Klarskov B, Kristiansen VB, Callesen T, Schulze S, Kehlet H, Rosenberg J. Multi-regional local anesthetic infiltration during laparoscopic cholecystectomy in patients receiving prophylactic multi-modal analgesia: A randomized, double-blinded, placebo-controlled study. *Anesth Analg* 1999; **89**: 1017-1024 [PMID: 10512282 DOI: 10.1097/00000539-199910000-00036]

13 **Hall H**, Leach A. Paravertebral block in the management of liver capsule pain after blunt trauma. *Br J Anaesth* 1999; **83**: 819-821 [PMID: 10690152 DOI: 10.1093/bja/83.5.819]

14 **Basak F**, Hasbahceci M, Sisik A, Acar A, Ozel Y, Canbak T, Yucel M, Ezberci F, Bas G. Glisson's capsule cauterisation is associated with increased postoperative pain after laparoscopic cholecystectomy: A prospective case-control study. *Ann R Coll Surg Engl* 2017; **99**: 485-489 [PMID: 28660823 DOI: 10.1308/rcsann.2017.0068]

15 **Iwasaki M**, Edmondson M, Sakamoto A, Ma D. Anesthesia, surgical stress, and "long-term" outcomes. *Acta Anaesthesiol Taiwan* 2015; **53**: 99-104 [PMID: 26235899 DOI: 10.1016/j.aat.2015.07.002]

16 **Krikri A**, Alexopoulos V, Zoumakis E, Katsaronis P, Balafas E, Kouraklis G, Karayannacos PE, Chrousos GP, Skalkeas G. Laparoscopic vs. open abdominal surgery in male pigs: Marked differences in cortisol and catecholamine response depending on the size of surgical incision. *Hormones (Athens)* 2013; **12**: 283-291 [PMID: 23933697 DOI: 10.14310/horm.2002.1412]

17 **Kapritsou M**, Papathanassoglou ED, Bozas E, Korkolis DP, Konstantinou EA, Kaklamanos I, Giannakopoulou M. Comparative Evaluation of Pain, Stress, Neuropeptide Y, ACTH, and Cortisol Levels Between a Conventional Postoperative Care Protocol and a Fast-Track Recovery Program in Patients Undergoing Major Abdominal Surgery. *Biol Res Nurs* 2017; **19**: 180-189 [PMID: 28198198 DOI: 10.1177/1099800416682617]

18 **Iverson RE**, Gomez JL. Deep venous thrombosis: prevention and management. *Clin Plast Surg* 2013; **40**: 389-398 [PMID: 23830747 DOI: 10.1016/j.cps.2013.04.002]

19 **Lewis CE**. Preventing postoperative pneumonia. *JAMA Surg* 2014; **149**: 919 [PMID: 25054221 DOI: 10.1001/jamasurg.2014.1249]

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ORIGINAL ARTICLE

Observational Study

Analysis of 72 patients with colorectal high-grade neuroendocrine neoplasms from three Chinese hospitals

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Abstract

BACKGROUND

Colorectal high-grade neuroendocrine neoplasms (HGNENs) are rare and constitute less than 1% of all colorectal malignancies. Based on their morphological differentiation and proliferation identity, these neoplasms present heterogeneous clinicopathologic features. Opinions regarding treatment strategies for and improvement of the clinical outcomes of these patients remain controversial.

AIM

To delineate the clinicopathologic features of and explore the prognostic factors for this rare malignancy.

METHODS

This observational study reviewed the data of 72 consecutive patients with colorectal HGNENs from three Chinese hospitals between 2000 and 2019. The clinicopathologic characteristics and follow-up data were carefully collected from

research.

Data sharing statement: No additional data are available.

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their medical records, outpatient reexaminations, and telephone interviews. A survival analysis was conducted to evaluate their outcomes and to identify the prognostic factors for this disease.

RESULTS

According to the latest recommendations for the classification and nomenclature of colorectal HGNENs, 61 (84.7%) patients in our cohort had poorly differentiated neoplasms, which were categorized as high-grade neuroendocrine carcinomas (HGNECs), and the remaining 11 (15.3%) patients had well differentiated neoplasms, which were categorized as high-grade neuroendocrine tumors (HGNETs). Most of the neoplasms (63.9%) were located at the rectum. More than half of the patients (51.4%) presented with distant metastasis at the date of diagnosis. All patients were followed for a median duration of 15.5 mo. In the entire cohort, the median survival time was 31 mo, and the 3-year and 5-year survival rates were 44.3% and 36.3%, respectively. Both the univariate and multivariate analyses demonstrated that increasing age, HGNEC type, and distant metastasis were risk factors for poor clinical outcomes.

CONCLUSION

Colorectal HGNENs are rare and aggressive malignancies with poor clinical outcomes. However, patients with younger age, good morphological differentiation, and without metastatic disease can have a relatively favorable prognosis.

Key words: Colon; Rectum; Neuroendocrine; Neoplasm; Metastasis; Prognosis

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Core tip: Colorectal high-grade neuroendocrine neoplasms (HGNENs) are aggressive malignancies with an extremely low incidence. Many issues, such as their classification and therapy strategies, have been controversial for a long time. We conducted this study to delineate their clinicopathologic features and clinical outcomes. There is a trend to categorize colorectal HGNENs with good morphological differentiation as a subgroup different from high-grade neuroendocrine carcinomas in the newest World Health Organization classification. We introduced this classification into our study and compared the prognoses of different subgroups.

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INTRODUCTION

Colorectal high-grade neuroendocrine neoplasm (HGNEN) is a rare malignancy originating from neuroendocrine cells in the colon and rectum, and it constitutes less than 1% of all colorectal carcinomas[1,2]. Based on the 2010 World Health Organization (WHO) classification, neuroendocrine neoplasms with a high mitotic rate (over 20/10 high power fields) or Ki-67 labeling index (over 20%) were defined as HGNEN or neuroendocrine neoplasm (NEN) G3, including small-cell and large-cell subtypes. All colorectal high-grade neuroendocrine neoplasms (HGNENs) were regarded as poorly differentiated. Therefore, the term HGNEN was synonymous with high-grade neuroendocrine carcinoma (HGNEC)[3]. However, the histological grade is not always in line with the degree of morphological differentiation; in some patients, tumors are high grade but present good differentiation[4]. These patients show significantly different tumor biology, behavior, and prognosis compared with those with poorly differentiated HGNENs. Therefore, the consensus has been that colorectal HGNENs are not a homogenous entity[5]. In the 2017 WHO classification for pancreatic NEN, neuroendocrine tumor G3 (NET G3) was put forward as a new term and was defined

as a new subgroup of pancreatic HGNENs with good differentiation, whereas neuroendocrine carcinoma (NEC) only refers to poorly differentiated G3 pancreatic HGNENs. HGNEN or NEN G3 included both NET G3 and NEC. There is a trend towards introducing this new classification system into the management of colorectal HGNENs in the near future[6]. At the 16th annual European Neuroendocrine Tumor Society (ENETS) Conference in 2019, Professor Aurel Perren presented "New WHO Classification-Important News", stating that the terminology of NET G3 is extended to other primary sites, including the colon and rectum. According to the latest updates on the classification and grading of colorectal NENs, all cases in the present study were categorized as well-differentiated subtype (NET G3) and poorly differentiated subtype (NEC) on the basis of histomorphology.

Similar to small cell lung cancer, colorectal HGNENs are highly aggressive with a dismal prognosis, and over half of the patients have distant metastasis at the time of diagnosis[7]. The clinical manifestations are nonspecific, including hematochezia, abdominal pain, changes in bowel habits, and obstruction. Carcinoid syndrome is rare because most colorectal HGNENs are nonfunctional[8]. For early and small lesions, colorectal HGNENs usually present typical endoscopic features that are different from colorectal adenocarcinomas. They arise in the deeper layers of the intestinal mucosa and appear as smooth sessile lesions with normal overlying mucosa. Yellow mucosal discolouration might be observed in cases with positive expression of chromogranin[9,10]. However, most cases present large and advanced lesions at the date of diagnosis, and these lesions show no significantly different endoscopic presentations compared with other colorectal tumors. Therefore, it is difficult to distinguish HGNENs from common adenocarcinoma by a routine diagnostic technique. Immunohistochemical evaluation is necessary since HGNENs have special neuroendocrine markers, such as synaptophysin, chromogranin A, and neuron-specific enolase[11]. Due to the extremely low incidence rate of HGNENs, there are very few related prospective clinical studies; most studies are case reports or retrospective studies with small samples from single institutions in Western countries. As a consequence, no standard treatment guidelines have been made, and the efficacy of surgery and chemotherapy remains controversial.

Since most previous studies are case reports or small sample reports from single centers and Western countries, we conducted a multicenter prospective study and enrolled 72 patients from three different Chinese hospitals, aiming to improve our understanding of the clinicopathologic features and oncologic prognosis of patients with colorectal HGNENs.

MATERIALS AND METHODS

Patients

Our study was approved by the ethics committee of the National Cancer Center and was performed according to the Helsinki Declaration of the World Medical Association. All patients signed an informed consent form before the study. We reviewed the electronic medical records from three different Chinese institutions and enrolled 72 consecutive colorectal HGNEN patients from January 2000 to January 2019, including 47 from the Cancer Hospital Chinese Academy of Medical Sciences, 20 from China-Japan Friendship Hospital, and 5 from Beijing Hospital. Information regarding patient demographics, clinicopathologic features, treatment modalities, and oncologic outcomes was carefully collected and analyzed. All cases were definitively diagnosed with colorectal HGNEN through colonoscopy, abdominal and pelvic enhanced computed tomography scans, tissue biopsy, pathological examination, and immunohistochemical evaluation. All patients were confirmed to have a high mitotic rate (over 20/10 high power fields) and/or Ki-67 labeling index (over 20%). Moreover, cases with a component of adenocarcinoma or squamous carcinoma were excluded.

Statistical analysis

Our study received statistical review by one biomedical statistician in our institution. All data were analyzed using the Statistical Package for the Social Sciences (SPSS version 24.0, IBM Corp., Armonk, NY, United States). Quantitative data that followed the normal distribution are expressed as the median ± standard deviation, while quantitative data that did not follow the normal distribution are expressed as median and range. Qualitative data and ordinal data are presented as the number of cases and percentages. Survival time was defined as the time interval between the date of pathological diagnosis and death. Survival rates were calculated by the Kaplan-Meier method and further compared through the log-rank test. In addition, multivariate

analysis was performed using the Cox proportional hazards regression model to identify the independent prognostic factors. A P-value < 0.05 was considered statistically significant.

RESULTS

Patients' characteristics

The patients' characteristics are presented in Table 1. A total of 72 patients with a median age of 59.5 years old (range, 18-82 years old), including 52 (72.2%) males and 20 (27.8%) females, were enrolled in our study. The average body mass index (BMI) was 23.8 ± 3.4 kg/m^2. The common symptoms were hematochezia (37, 51.4%), abdominal pain (23, 31.9%), changes in bowel habits (23, 31.9%), abdominal distention (5, 9.6%), weight loss (3, 4.2%), and anemia (2, 2.8%). Two patients were asymptomatic, and cancer was detected through routine health examinations. No patients had functional tumors or presented with carcinoid syndrome. The rectum (n = 46, 63.9%), especially low rectum, was the most common primary site. Among the 46 patients with rectal HGNENs, 28 (60.9%) were located in the low rectum. More than half of the patients (51.4%) presented metastatic diseases at the date of diagnosis, and the liver and distant lymph nodes were the two most common metastatic sites.

Pathological features

The pathological features and immunohistochemical results are listed in Table 2. Of the 72 patients, 61 (84.7%) had poorly differentiated tumors classified as NECs, and the remaining 11 patients had well differentiated tumors classified as NETs G3. Among the 61 NEC patients, 18 (29.5%) and 18 (29.5%) had large cell and small cell subtypes, respectively. Cancers of the remaining 25 (41%) patients were not further categorized in the medical records. Regarding the general shape of neoplasms in the 58 evaluable patients, one half were ulcerative, and the other half were the protruding type. All the patients received immunohistochemical evaluation, and the median value of the Ki67 index was 70% in our cohort. Synaptophysin, chromogranin, neuron-specific enolase, and CD 56 were positive in 94%, 57.6%, 64.3%, and 82.4%, respectively, of all evaluable cases. CDX-2 and TTF-1 were evaluated in 29 and 13 patients, respectively, and the positive rates were 62.1% and 15.4%, respectively. Extramural vascular invasion (EMVI) and perineural invasion were observed in 76.3% and 21.6% of evaluable patients, respectively.

Treatment management

Of the 35 patients without distant metastasis, 1 received only chemotherapy. This patient underwent a cycle of combination chemotherapy of cisplatin and etoposide and two cycles of single-agent irinotecan. However, the neoplasm progressed, and the patient died in the hospital 3 mo after the date of diagnosis. The other 34 patients underwent surgical resection of tumors, including 2 patients who underwent local excision. Six patients received surgery alone. Five patients received neoadjuvant therapy, and all responded to therapy, with one achieving a pathologic complete response and surviving free from recurrence for 14 mo by the end of follow-up. Twenty-eight patients received adjuvant therapy. Five patients received both neoadjuvant and adjuvant treatment (Table 3).

Of the 37 patients with distant metastasis at the date of diagnosis, 17 underwent surgery and received primary site resection, 17 received palliative chemotherapy and/or radiotherapy alone, and 3 did not receive any oncological treatment. The details of palliative chemotherapy were evaluable for 28 cases. Twenty-eight cases received first-line palliative chemotherapy, and 9 (32.1%) cases were responsive. Twelve of 28 (42.9%) patients received fluorouracil (5-FU)-based chemotherapy [capecitabine plus oxaliplatin (n = 5), oxaliplatin, leucovorin, and fluorouracil (n = 3), oxaliplatin plus fluorouracil (n = 1), irinotecan plus tegafur/gimeracil/oteracil potassium (S-1) (n = 1), capecitabine plus temozolomide (TemCap) (n = 1), and S-1 (n = 1)], and 1 patient (8.3%) responded. The remaining 16 (57.1%) patients received platinum-based chemotherapy [cisplatin plus etoposide (EP) (n = 14), oxaliplatin plus etoposide (n = 1), carboplatin plus etoposide (n = 1)], and 8 (50%) cases responded. Thirteen and 9 cases received second-line and third-line palliative chemotherapy, and the responsive rates were 23.1% and 22.2%, respectively.

Of the three patients who did not receive any oncological treatment, one survived for only 1 mo, one survived for 3 mo, and one was lost to follow-up.

Oncological prognosis

All patients were followed for a median duration of 15.5 mo (range, 1-190 mo). A median survival of 31 mo was achieved in the whole cohort, and the 3-year and 5-year

Table 1 Patient characteristics

Characteristic	Patients (*n* = 72)
Sex, *n* (%)	
Male	52 (72.2)
Female	20 (27.8)
Age [yr, median (range)]	59.5 (18-82)
BMI (kg/m^2, mean ± SD)	23.8 ± 3.4
Symptoms, *n* (%)	
Hematochezia	37 (51.4)
Abdominal pain	23 (31.9)
Changes in bowel habits	23 (31.9)
Obstruction	12 (16.7)
Abdominal distention	5 (9.6)
Weight loss	3 (4.2)
Anemia	2 (2.8)
Carcinoid syndrome	0
Asymptomatic	2 (2.8)
Family history of cancer, *n* (%)	
Yes	11 (15.3)
No	60 (83.3)
Unrecorded	1 (1.4)
History of colorectal polyps, *n* (%)	
Yes	24 (33.3)
No	27 (37.5)
Unrecorded	21 (29.2)
Smoking history, *n* (%)	
Yes	28 (38.9)
No	42 (58.3)
Unrecorded	2 (2.8)
Drinking history, *n* (%)	
Yes	24 (33.3)
No	45 (62.5)
Unrecorded	3 (4.2)
Primary sites, *n* (%)	
Rectum	46 (63.9)
Rectosigmoid junction	2 (2.8)
Sigmoid	5 (6.9)
Descending colon	4 (5.6)
Transverse colon	2 (2.8)
Ascending colon	9 (12.5)
Cecum	4 (5.6)
Distance of tumor from the anal verge [(for rectal carcinoma, *n* = 46), *n* (%)]	
0-5 cm	28 (60.9)
5-10 cm	14 (19.4)
10-15 cm	2 (2.8)
Unrecorded	2 (2.8)
Tumor size [median (range), cm]	5.0 (1.0-15.0)
Tumor stage, *n* (%)	
I	4 (5.6)
II	4 (5.6)
III	27 (37.5)
IV	37 (51.4)
Site of distant metastases, *n* (%)	
Liver	27 (37.5)

Liver only	12 (16.6)
Distant lymph nodes	15 (20.8)
Peritoneum	5 (6.9)
Bone	5 (6.9)
Lung	1 (1.4)
Pancreas	1 (1.4)
Increase of pretreatment blood LDH, *n* (%)	
Yes	10 (13.9)
No	29 (40.3)
Unrecorded	33 (45.8)

BMI: Body mass index; SD: Standard deviation; LDH: Lactic dehydrogenase.

survival rates were 44.3% and 36.3%, respectively. A significantly decreased median survival of 13 mo was observed for the patients with metastatic disease. Since more than half of the patients without distant metastasis (67%) survived through the end of follow-up, the median survival of these patients could not be calculated. Univariate analysis demonstrated that age ($P < 0.001$), pathologic type ($P = 0.033$), neoplasm macroscopic type ($P = 0.037$), distant metastasis ($P < 0.001$), positive EMVI ($P = 0.047$), elevation of pretreatment serum lactate dehydrogenase ($P = 0.015$), and resection of the primary site ($P < 0.001$) were associated with the overall survival of patients with colorectal HGNEC (Figure 1). For unclear reasons, no significant survival advantage was found in patients with a low Ki-67 index (<55%), as reported in previous studies. To identify the independent prognostic factors, multivariate analysis was subsequently performed. Based on previous studies and knowledge, we enrolled 6 variables: Gender, age, tumor location, pathological type, distant metastasis, and resection of the primary site. Given the missing data for the pretreatment level of serum lactate dehydrogenase, tumor macroscopic type, EMVI, and Ki-67 index, these variables were not included in the multivariate analysis. Consequently, age ≥ 70 [hazard ratio (HR) = 3.926, 95% confidence interval (CI): 1.740-8.858, $P = 0.001$], pathologic type of NEC (HR = 6.647, 95%CI: 1.759-25.119, $P = 0.005$), and distant metastasis (HR = 6.356, 95%CI: 2.543-15.889, $P < 0.001$) were confirmed to be independent risk factors for poor prognosis (Table 4).

DISCUSSION

Colorectal HGNEN is an extremely rare malignancy with an incidence rate ranging from 1 to 2 per million, constituting less than 1% of all colorectal malignancies. However, its incidence rate has been increasing in the past decades, and the reported annual increase rate ranges from 2.2% to 9.4%[12,13]. Moreover, its clinical prognosis is much worse compared to colorectal adenocarcinoma. It seems that the advances in the study of colorectal adenocarcinoma did not benefit the prevention and treatment of colorectal HGNENs. Our multicenter retrospective study delineated the clinicopathologic features, clinical outcomes, and prognostic factors for this rare tumor.

In previous reports, rectal HGNEN was the most frequent and accounted for 26.5% to 64% of all colorectal HGNEN cases[13,14]. In line with these prior studies, 63.9% of the cases in our study were rectal HGNEN. More notably, 60.9% of these rectal cases were located in the low rectum. Similar to small cell lung cancer, colorectal HGNEN presented a high degree of malignancy and a high risk of distant metastasis compared to colorectal adenocarcinoma. More than half of the patients had metastatic disease at diagnosis. One investigation based on the Survey of Epidemiology and End Results database analyzed the data from 1367 cases of colorectal HGNEN and 72533 cases of colorectal adenocarcinoma. A significantly higher rate of distant metastases was observed in the HGNEN group (57.9%) than in the adenocarcinoma group (25.2%)[13]. In the present study, 51.4% of the patients presented with distant metastasis at the date of diagnosis. The liver and distant lymph nodes were the most common sites of metastases. There was a trend showing that patients with colonic HGNEN (61.5%) were more likely to develop metastatic disease than those with rectal HGNEN (45.7%). This might be because patients with rectal HGNEN could have rectal bleeding and changes in bowel habits at a relatively early stage, which promoted the early detection of cancer[15]. In contrast to small cell lung cancer, the clinical presentation of colorectal HGNEN is not specific. Carcinoid syndromes can hardly be

Table 2 Pathological features

Histology, *n* (%)	Patients (*n* = 72)
NEC	61 (84.7)
NET G3	11 (15.3)
General classification of tumor, *n* (%)	
Ulcerative type	29 (40.3)
Protruding type	29 (40.3)
Unrecorded	14 (19.4)
Synaptophysin, *n* (%)	
Positive	63 (87.5)
Negative	4 (5.6)
Unrecorded	5 (6.9)
Chromogranin, *n* (%)	
Positive	38 (52.8)
Negative	28 (38.9)
Unrecorded	6 (8.3)
Neuron specific enolase, *n* (%)	
Positive	9 (12.5)
Negative	5 (6.9)
Unrecorded	58 (80.6)
CD56, *n* (%)	
Positive	42 (58.3)
Negative	9 (12.5)
Unrecorded	21 (29.2)
CDX-2, *n* (%)	
Positive	18 (25)
Negative	11 (15.3)
Unrecorded	43 (59.7)
TTF-1, *n* (%)	
Positive	2 (2.8)
Negative	11 (15.3)
Unrecorded	59 (81.9)
Ki 67 (median, range)	70% (25%-95%)
EMVI, *n* (%)	
Yes	29 (40.3)
No	9 (12.5)
Unrecorded	34 (47.2)
Perineural invasion, *n* (%)	
Yes	8 (11.1)
No	29 (40.3)
Unrecorded	35 (48.6)

NEC: Neuroendocrine carcinoma; NET: Neuroendocrine tumor; EMVI: Extramural vascular invasion.

seen as most of these tumors are nonfunctional and cannot secrete 5-hydroxytryptamine[16]. To date, only several cases with hormonal symptoms have been reported in the literature. These patients presented symptoms such as facial flushing, sweating, and diarrhea due to excessive production of hormones[15,17]. In our study, no patients presented with carcinoid syndromes. In most cases, there was no difference in the symptoms or signs between colorectal HGNEN and adenocarcinoma. Hematochezia, abdominal pain, and changes in bowel habits were the most common presentations.

Given the difficulty of distinguishing colorectal HGNEN from adenocarcinoma through clinical manifestation, pathological examination and immunohistochemical evaluation are necessary. In the 2010 WHO classification for gastroenteropancreatic NEN, NENs with a mitotic count greater than 20 per high power field or Ki-67 index

Table 3 Management of patients with localized disease

Treatment strategy	Patients (*n* = 35)
Nonsurgical treatment	1
Surgery alone	6
Surgery + adjuvant treatment	23
Neoadjuvant treatment + surgery + adjuvant treatment	5

greater than 20% are considered poorly differentiated, including small cell and large cell subtypes. Therefore, patients who meet this standard are diagnosed with G3 NEC or HGNEC[18]. However, the histological grade is not always in line with the degree of tumor differentiation[19]. There are some HGNEN cases that show good differentiation, biological behavior similar to that of G2 NETs, and good prognosis. In the 2017 WHO classification for pancreatic NET, well-differentiated G3 pancreatic NENs were categorized as a new subgroup called NET G3, whereas NEC only refers to poorly differentiated G3 pancreatic NENs. Both NET G3 and NEC together were referred to as NEN G3[20,21]. There is a general tendency for this new grading system to be introduced into the classification of colorectal NETs. In the 16th annual ENETS Conference in 2019, Professor Aurel Perren presented "New WHO Classification-Important News", stating that the terminology of NET G3 is extended to other primary sites, including the colon and rectum. Based on the latest updates on classification and grading of colorectal NENs, all cases in the present study were categorized as well-differentiated subtype (NET G3) and poorly differentiated subtype (NEC) on the basis of histomorphology. However, it was challenging to distinguish NET G3 from NEC based on morphology differentiation alone in many cases. Therefore, genetic status and proliferative activity can be referenced in the updated classification. Cases with mutations of *KRAS*, *BRAF*, *p53*, and *Rb1*, or with Ki67 index greater than 70%-80% tended to be classified as NEC. A total of 61 cases in our research were confirmed to be NEC. The remaining 11 cases were categorized as NET G3 and constituted 15.3% of all cases, which was higher than previous reports (5.5%-8.7%)[19,22].

Colorectal HGNEN can present characteristic manifestations through immunohistochemical examination. In one retrospective study of 100 colorectal HGNEN cases, synaptophysin was the most sensitive biomarker in the diagnosis of colorectal HGNEN and showed a sensitivity of 93%, which was evidently higher than that of chromogranin (58%) and neuron-specific enolase (87%)[23]. Similarly, synaptophysin demonstrated the highest sensitivity (94%), followed by CD56 (82.4%), neuron-specific enolase (64.3%), and chromogranin (57.6%) in the evaluable cases in our research. Moreover, EMVI was extremely common in colorectal HGNEN and was positive in 76.3% of the evaluable patients. This might help explain the fact that colorectal HGNEN was more prone to distant metastasis at the time of diagnosis.

Due to the rarity of colorectal HGNEN, most published studies are limited to case reports or retrospective studies with small samples. The treatment regimen for this malignancy remains controversial because there is no acknowledged therapeutic schedule. The present treatment regimens are usually extrapolated from evidence on small cell lung cancer and colorectal adenocarcinoma. For patients with localized disease, surgery remains the most common choice in most cases in clinical practice. However, it is still debatable whether patients can benefit from the surgical resection of primary tumors[24,25]. In one retrospective report with 126 colorectal HGNEC patients, surgery did not offer a survival benefit for patients without metastatic disease (median survival, 27.4 mo with surgery *vs* 20.3 mo without surgery, P = 0.17)[15]. In another retrospective study based on the Survey of Epidemiology and End Results database, the survival outcomes for patients who received surgery differed by histologic subcategory. In the non-small cell group, surgery improved the oncological prognosis (median survival, 21 mo with surgery *vs* 6 mo without surgery, $P < 0.001$). In the small cell group, surgery was not associated with superior outcomes (median survival, 18 mo with surgery *vs* 14 mo without surgery, $P = 0.95$). This finding is in line with the experience for small-cell lung cancer[13]. In the present study, 34 of 35 (97.1%) patients with localized disease received radical surgery. As only 1 patient did not receive surgery, we could not evaluate the efficacy of surgery. Systemic chemotherapy is regarded as the mainstay for treatment of patients with metastatic disease. Based on the 2010 WHO classification, for all patients with NENs of grade G3, the EP regimen was recommended as the choice for palliative first-line chemotherapy. However, based on the newest classification and grading for NENs

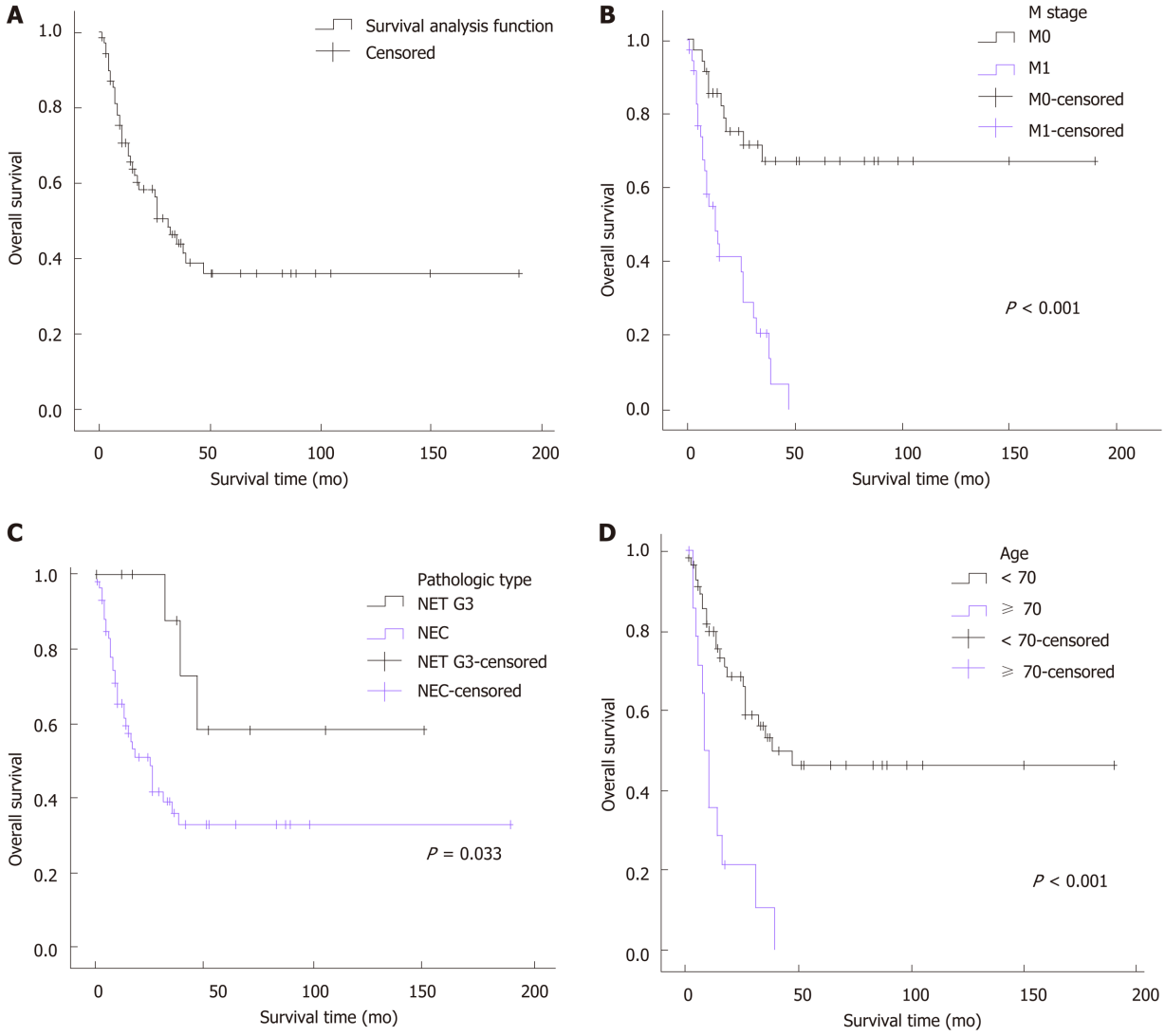

Figure 1 Univariate analysis of the survival rates of colorectal high-grade neuroendocrine neoplasm. A: Overall survival rate of the entire cohort; B: The cohorts with or without metastatic disease; C: The cohorts categorized by pathologic type; D: The cohorts of age < 70 or ≥ 70. NEC: Neuroendocrine carcinoma; NET: Neuroendocrine tumor; NET G3: Neuroendocrine tumor G3.

G3, the EP regimen was recommended only for patients with NECs, while patients with NETs G3 might benefit from the medical strategy used in NETs G2. Therefore, the TemCap regimen (temozolomide plus capecitabine) is now recommended as a first-line palliative treatment for NETs G3. However, both the retrospective and prospective data related to palliative chemotherapy for NETs G3 were scarce[26]. In the present study, 28 cases received palliative first-line chemotherapy, and the overall response rate was 32.1%. Twelve of 28 (42.9%) patients received 5-FU-based chemotherapy, and 1 (8.3%) patient responded. The remaining 16 (57.1%) patients received platinum-based chemotherapy and showed a response rate of 50%, which is in line with previously reported response rates (ranging from 30% to 50%)[26]. The statistical analysis demonstrated that HGNENs were significantly more sensitive to platinum-based chemotherapy than fluorouracil 5-FU-based chemotherapy (P = 0.039).

Many previous studies of prognosis have delineated poor clinical outcomes of colorectal HGNENs, with a median overall survival (OS) ranging from 9 mo to 20.6 mo, 3-year OS rates ranging from 8.7%-35%, and 5-year OS rates ranging from 8%-13.3%[2,15,17,25,27]. However, most of these reports only enrolled patients with NECs, and survival data for colorectal NETs G3 were scarce. To the best of our knowledge, our study has had the largest sample size enrolling both colorectal NECs and NETs G3 cases to date. As we included many cases with good differentiation, a better prognosis was observed in our cohort, with a median OS of 31 mo and 3-year and 5-year OS rates of 44.3% and 36.3%, respectively. Moreover, unlike previous studies that enrolled some cases diagnosed before 2000, all cases in our study were diagnosed

Table 4 Survival analysis of overall survival

Variable	Univariate analysis		Multivariate analysis	
	Median OS	*P*-value	HR (95%CI)	*P*-value
Sex		0.095	1.374 (0.664-2.845)	0.392
Male	39			
Female	18			
Age (yr)		<0.001	3.926 (1.740-8.858)	0.001
<70	47			
≥70	8			
Radical surgery		<0.001	0.778 (0.338-1.792)	0.555
Yes	39			
No	8			
Tumor location		0.386	1.592 (0.738-3.434)	0.236
Colon	15			
Rectum	35			
Gross type		0.037		
Ulcerative	18			
Protruding	imponderable			
Distant metastasis		<0.001	6.356 (2.543-15.889)	<0.001
Yes	13			
No	imponderable			
Pathologic type		0.033	6.647 (1.759-25.119)	0.005
NEC	25			
NET G3	imponderable			
Ki67 index		0.893		
<55%	39			
≥55%	35			
EMVI		0.047		
Yes	26			
No	Imponderable			
Perineural invasion		0.944		
Yes	26			
No	39			
Pretreatment bloodLDH level		0.015		
Elevated	7			
Not elevated	26			

OS: Overall survival; CI: Confidence interval; NEC: Neuroendocrine carcinoma; NET: Neuroendocrine tumor; EMVI: Extramural vascular invasion; LDH: Lactic dehydrogenase; HR: Hazard ratio; NET G3: Neuroendocrine tumor G3.

after 2000. The advances in the management of colorectal NENs might contribute to the improved clinical outcomes that were observed in our reports. Both the univariate analysis (P = 0.033) and multivariate analysis (P = 0.005) demonstrated a better prognosis of NETs G3 compared to NECs. For patients with NETs G3, the median OS could not be calculated as over half of the patients survived through the end of follow-up, and the 3-year and 5-year OS rates were 87.5% and 58.3%, respectively. For patients with NECs, the median OS was 25 mo, and the 3-year and 5-year OS rates were 36.4% and 33.1%, respectively. Significant differences in clinical outcomes between NETs G3 and NECs showed that colorectal NETs G3 were less malignant and should not be treated with the same strategies as NECs. Metastatic disease is also an important prognostic factor. In previous reports, 57.9%-67% of patients with colorectal HGNEN presented with distant metastasis at the date of diagnosis[13,15]. However, they accounted for only 51.4% in our study, which might be another reason that our cohort showed a better prognosis than previous studies. These patients had a median OS of only 13 mo, with 3-year and 5-year OS rates of 20.9% and 0, respectively, which was significantly worse than patients without metastatic disease

based on both the univariate analysis ($P < 0.001$) and multivariate analysis ($P < 0.001$). In addition, we observed a strong trend towards a worse prognosis associated with increasing age. Patients over 70 years old showed a much poorer median survival time (7 mo in patients ≥ 70 *vs* 47 mo in patients < 70, $P < 0.001$). This trend was also observed in several prior reports, although the underlying mechanisms have not been well illuminated[13,23]. Elderly patients are usually in poor physical conditions due to their comorbidities, such as diabetes, coronary heart disease, and hepatic and renal dysfunction. This can both decrease their antitumor abilities and constrain the choices of therapy strategies, which subsequently leads to a poor prognosis. Moreover, univariate analyses demonstrated that patients with ulcerative neoplasms, EMVI, and elevated pretreatment blood LDH levels were associated with worse clinical outcomes. However, we did not enroll these factors in the multivariate analysis since these data were not available for all of our cases. Further studies can explore the association between these variables and prognosis so that we can predict survival outcomes through pretreatment examinations.

Our study had several limitations. First, it is a retrospective study, and the bias from patient selection and information collection is unavoidable. Second, the period of our study is within a span of nearly 20 years, the nomenclature and classification of colorectal NETs has been changing, and the early pathological reports are not as normative as they are now. This leads to the lack of vital information, such as the Ki-67 index and pathological type (small cell or large cell), in some patients and makes it difficult to evaluate their value in predicting prognosis.

In conclusion, colorectal HGNENs are rare and heterogeneous groups of malignancies. They present distinct clinicopathologic characteristics with colorectal adenocarcinoma and show a dismal prognosis. Patients with pathologic type NETs G3, younger age, and without distant metastasis might have relatively good clinical outcomes.

ARTICLE HIGHLIGHTS

Research background

Colorectal high-grade neuroendocrine neoplasms (HGNENs) are aggressive malignancies with a dismal prognosis. Due to the rarity of this disease, there are still no related large multicenter prospective randomized studies. Therefore, no standard management recommendations have been established.

Research motivation

Most previous reports are case reports and retrospective studies with small samples from single center of Western countries, and few data from multicenter studies or China can be found. Moreover, there is a trend that colorectal HGNENs will be classified as neuroendocrine carcinomas (NECs) and neuroendocrine tumors G3 (NETs G3) based on their morphological differentiation. In prior studies, all colorectal HGNENs were considered NECs.

Research objectives

Based on the latest classification and grading recommendations, we aimed to improve our understanding of this rare disease through multicenter data from China.

Research methods

We performed an observational study and enrolled patients with colorectal HGNENs from three Chinese hospitals. Information regarding the clinicopathologic features and clinical outcomes was collected and delineated. The prognostic factors were analyzed using the Kaplan-Meier method and the Cox proportional hazards regression model.

Research results

Colorectal HGNENs are highly aggressive, and more than half of the patients have developed distant metastasis at the date of diagnosis. It is difficult to distinguish HGNENs from adenocarcinoma through clinical presentations, and immunohistochemical evaluation is necessary. Survival analysis demonstrated that colorectal NETs G3 had a significantly better prognosis than NECs. Therefore, colorectal HGNENs were not a homogenous group of malignancies, and colorectal NETs G3 should be treated with different strategies from NECs. Moreover, increasing age and distant metastasis were statistically confirmed to be independent risk factors for poor clinical outcomes.

Research conclusions

Colorectal HGNENs are aggressive and heterogeneous groups of malignancies. Patients with younger age, good morphological differentiation, and without metastatic disease can have a relatively favorable prognosis.

Research perspectives

More large prospective multicenter clinical studies need to be performed so that standard

management recommendations can be established. Moreover, colorectal NETs G3 is an emerging term for colorectal HGNENs with good differentiation and that present significantly different biological behavior from NECs. Distinguishing colorectal NETs G3 from NECs is not always easy. It is imperative to further explore their respective molecular mechanisms and genetic changes so that better diagnostic and treatment strategies can be achieved in the future.

REFERENCES

1 **Wu Z**, Yu D, Zhao S, Gao P, Song Y, Sun Y, Chen X, Wang Z. The efficacy of chemotherapy and operation in patients with colorectal neuroendocrine carcinoma. *J Surg Res* 2018; **225**: 54-67 [PMID: 29605035 DOI: 10.1016/j.jss.2017.12.035]

2 **Bernick PE**, Klimstra DS, Shia J, Minsky B, Saltz L, Shi W, Thaler H, Guillem J, Paty P, Cohen AM, Wong WD. Neuroendocrine carcinomas of the colon and rectum. *Dis Colon Rectum* 2004; **47**: 163-169 [PMID: 15043285 DOI: 10.1007/s10350-003-0038-1]

3 **Caplin M**, Sundin A, Nillson O, Baum RP, Klose KJ, Kelestimur F, Plöckinger U, Papotti M, Salazar R, Pascher A; Barcelona Consensus Conference participants. ENETS Consensus Guidelines for the management of patients with digestive neuroendocrine neoplasms: colorectal neuroendocrine neoplasms. *Neuroendocrinology* 2012; **95**: 88-97 [PMID: 22261972 DOI: 10.1159/000335594]

4 **Garcia-Carbonero R**, Sorbye H, Baudin E, Raymond E, Wiedenmann B, Niederle B, Sedlackova E, Toumpanakis C, Anlauf M, Cwikla JB, Caplin M, O'Toole D, Perren A; Vienna Consensus Conference participants. ENETS Consensus Guidelines for High-Grade Gastroenteropancreatic Neuroendocrine Tumors and Neuroendocrine Carcinomas. *Neuroendocrinology* 2016; **103**: 186-194 [PMID: 26731334 DOI: 10.1159/000443172]

5 **Vélayoudom-Céphise FL**, Duvillard P, Foucan L, Hadoux J, Chougnet CN, Leboulleux S, Malka D, Guigay J, Goere D, Debaere T, Caramella C, Schlumberger M, Planchard D, Elias D, Ducreux M, Scoazec JY, Baudin E. Are G3 ENETS neuroendocrine neoplasms heterogeneous? *Endocr Relat Cancer* 2013; **20**: 649-657 [PMID: 23845449 DOI: 10.1530/ERC-13-0027]

6 **Sorbye H**, Baudin E, Borbath I, Caplin M, Chen J, Cwikla JB, Frilling A, Grossman A, Kaltsas G, Scarpa A, Welin S, Garcia-Carbonero R; ENETS 2016 Munich Advisory Board Participants. Unmet Needs in High-Grade Gastroenteropancreatic Neuroendocrine Neoplasms (WHO G3). *Neuroendocrinology* 2019; **108**: 54-62 [PMID: 30153658 DOI: 10.1159/000493318]

7 **Koenig A**, Krug S, Mueller D, Barth PJ, Koenig U, Scharf M, Ellenrieder V, Michl P, Moll R, Homayunfar K, Kann PH, Stroebel P, Gress TM, Rinke A. Clinicopathological hallmarks and biomarkers of colorectal neuroendocrine neoplasms. *PLoS One* 2017; **12**: e0188876 [PMID: 29232390 DOI: 10.1371/journal.pone.0188876]

8 **Starzyńska T**, Londzin-Olesik M, Bałdys-Waligórska A, Bednarczuk T, Blicharz-Dorniak J, Bolanowski M, Boratyn-Nowicka A, Borowska M, Cichocki A, Ćwikła JB, Deptała A, Falconi M, Foltyn W, Handkiewicz-Junak D, Hubalewska-Dydejczyk A, Jarząb B, Junik R, Kajdaniuk D, Kamiński G, Kolasińska-Ćwikła A, Kowalska A, Król R, Królicki L, Kunikowska J, Kuśnierz K, Lampe P, Lange D, Lewczuk-Myślicka A, Lewiński A, Lipiński M, Marek B, Nasierowska-Guttmejer A, Nowakowska-Duława E, Pilch-Kowalczyk J, Remiszewski P, Rosiek V, Ruchała M, Siemińska L, Sowa-Staszczak A, Steinhof-Radwańska K, Strzelczyk J, Sworczak K, Syrenicz A, Szawłowski A, Szczepkowski M, Wachuła E, Zajęcki W, Zemczak A, Zgliczyński W, Kos-Kudła B. Colorectal neuroendocrine neoplasms - management guidelines (recommended by the Polish Network of Neuroendocrine Tumours). *Endokrynol Pol* 2017; **68**: 250-260 [PMID: 28540975 DOI: 10.5603/EP.2017.0019]

9 **Basuroy R**, Haji A, Ramage JK, Quaglia A, Srirajaskanthan R. Review article: the investigation and management of rectal neuroendocrine tumours. *Aliment Pharmacol Ther* 2016; **44**: 332-345 [PMID: 27302838 DOI: 10.1111/apt.13697]

10 **Chablaney S**, Zator ZA, Kumta NA. Diagnosis and Management of Rectal Neuroendocrine Tumors. *Clin Endosc* 2017; **50**: 530-536 [PMID: 29207857 DOI: 10.5946/ce.2017.134]

11 **Kyriakopoulos G**, Mavroeidi V, Chatzellis E, Kaltsas GA, Alexandraki KI. Histopathological, immunohistochemical, genetic and molecular markers of neuroendocrine neoplasms. *Ann Transl Med* 2018; **6**: 252 [PMID: 30069454 DOI: 10.21037/atm.2018.06.27]

12 **Kang H**, O'Connell JB, Leonardi MJ, Maggard MA, McGory ML, Ko CY. Rare tumors of the colon and rectum: a national review. *Int J Colorectal Dis* 2007; **22**: 183-189 [PMID: 16845516 DOI: 10.1007/s00384-006-0145-2]

13 **Shafqat H**, Ali S, Salhab M, Olszewski AJ. Survival of patients with neuroendocrine carcinoma of the colon and rectum: a population-based analysis. *Dis Colon Rectum* 2015; **58**: 294-303 [PMID: 25664707 DOI: 10.1097/DCR.0000000000000298]

14 **Aytac E**, Ozdemir Y, Ozuner G. Long term outcomes of neuroendocrine carcinomas (high-grade neuroendocrine tumors) of the colon, rectum, and anal canal. *J Visc Surg* 2014; **151**: 3-7 [PMID: 24412088 DOI: 10.1016/j.jviscsurg.2013.12.007]

15 **Smith JD**, Reidy DL, Goodman KA, Shia J, Nash GM. A retrospective review of 126 high-grade neuroendocrine carcinomas of the colon and rectum. *Ann Surg Oncol* 2014; **21**: 2956-2962 [PMID: 24763982 DOI: 10.1245/s10434-014-3725-3]

16 **Anthony LB**, Strosberg JR, Klimstra DS, Maples WJ, O'Dorisio TM, Warner RR, Wiseman GA, Benson AB, Pommier RF; North American Neuroendocrine Tumor Society (NANETS). The NANETS consensus guidelines for the diagnosis and management of gastrointestinal neuroendocrine tumors (nets): well-differentiated nets of the distal colon and rectum. *Pancreas* 2010; **39**: 767-774 [PMID: 20664474 DOI: 10.1097/MPA.0b013e3181ec1261]

17 **Kim ST**, Ha SY, Lee J, Hong SN, Chang DK, Kim YH, Park YA, Huh JW, Cho YB, Yun SH, Lee WY, Kim HC, Park YS. The Clinicopathologic Features and Treatment of 607 Hindgut Neuroendocrine Tumor (NET) Patients at a Single Institution. *Medicine (Baltimore)* 2016; **95**: e3534 [PMID: 27175651 DOI: 10.1097/MD.0000000000003534]

18 **Cives M**, Strosberg JR. Gastroenteropancreatic Neuroendocrine Tumors. *CA Cancer J Clin* 2018; **68**: 471-487 [PMID: 30295930 DOI: 10.3322/caac.21493]

19 **Milione M**, Maisonneuve P, Spada F, Pellegrinelli A, Spaggiari P, Albarello L, Pisa E, Barberis M, Vanoli A, Buzzoni R, Pusceddu S, Concas L, Sessa F, Solcia E, Capella C, Fazio N, La Rosa S. The Clinicopathologic Heterogeneity of Grade 3 Gastroenteropancreatic Neuroendocrine Neoplasms:

Morphological Differentiation and Proliferation Identify Different Prognostic Categories. *Neuroendocrinology* 2017; **104**: 85-93 [PMID: 26943788 DOI: 10.1159/000445165]
20 **Kim JY**, Hong SM, Ro JY. Recent updates on grading and classification of neuroendocrine tumors. *Ann Diagn Pathol* 2017; **29**: 11-16 [PMID: 28807335 DOI: 10.1016/j.anndiagpath.2017.04.005]
21 **Guilmette JM**, Nosé V. Neoplasms of the Neuroendocrine Pancreas: An Update in the Classification, Definition, and Molecular Genetic Advances. *Adv Anat Pathol* 2019; **26**: 13-30 [PMID: 29912000 DOI: 10.1097/PAP.0000000000000201]
22 **Heetfeld M**, Chougnet CN, Olsen IH, Rinke A, Borbath I, Crespo G, Barriuso J, Pavel M, O'Toole D, Walter T; other Knowledge Network members. Characteristics and treatment of patients with G3 gastroenteropancreatic neuroendocrine neoplasms. *Endocr Relat Cancer* 2015; **22**: 657-664 [PMID: 26113608 DOI: 10.1530/ERC-15-0119]
23 **Conte B**, George B, Overman M, Estrella J, Jiang ZQ, Mehrvarz Sarshekeh A, Ferrarotto R, Hoff PM, Rashid A, Yao JC, Kopetz S, Dasari A. High-Grade Neuroendocrine Colorectal Carcinomas: A Retrospective Study of 100 Patients. *Clin Colorectal Cancer* 2016; **15**: e1-e7 [PMID: 26810202 DOI: 10.1016/j.clcc.2015.12.007]
24 **Brieau B**, Lepère C, Walter T, Lecomte T, Guimbaud R, Manfredi S, Tougeron D, Desseigne F, Lourenco N, Afchain P, El Hajbi F, Terris B, Rougier P, Coriat R. Radiochemotherapy Versus Surgery in Nonmetastatic Anorectal Neuroendocrine Carcinoma: A Multicenter Study by the Association des Gastro-Entérologues Oncologues. *Medicine (Baltimore)* 2015; **94**: e1864 [PMID: 26496341 DOI: 10.1097/MD.0000000000001864]
25 **Fields AC**, Lu P, Vierra BM, Hu F, Irani J, Bleday R, Goldberg JE, Nash GM, Melnitchouk N. Survival in Patients with High-Grade Colorectal Neuroendocrine Carcinomas: The Role of Surgery and Chemotherapy. *Ann Surg Oncol* 2019; **26**: 1127-1133 [PMID: 30706232 DOI: 10.1245/s10434-019-07203-3]
26 **Sorbye H**, Baudin E, Perren A. The Problem of High-Grade Gastroenteropancreatic Neuroendocrine Neoplasms: Well-Differentiated Neuroendocrine Tumors, Neuroendocrine Carcinomas, and Beyond. *Endocrinol Metab Clin North Am* 2018; **47**: 683-698 [PMID: 30098724 DOI: 10.1016/j.ecl.2018.05.001]
27 **Voong KR**, Rashid A, Crane CH, Minsky BD, Krishnan S, Yao JC, Wolff RA, Skibber JM, Feig BW, Chang GJ, Das P. Chemoradiation for High-grade Neuroendocrine Carcinoma of the Rectum and Anal Canal. *Am J Clin Oncol* 2017; **40**: 555-560 [PMID: 26237193 DOI: 10.1097/COC.0000000000000211]

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META-ANALYSIS

Bilateral *vs* unilateral placement of metal stents for inoperable high-grade hilar biliary strictures: A systemic review and meta-analysis

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Abstract

BACKGROUND

Bilateral *vs* unilateral biliary stenting is used for palliation in malignant biliary obstruction. No clear data is available to compare the efficacy and safety of bilateral biliary stenting over unilateral stenting.

AIM

To assess the efficacy and safety of bilateral *vs* unilateral biliary drainage in inoperable malignant hilar obstruction.

METHODS

PubMed, Embase, Scopus, and Cochrane databases, as well as secondary sources (bibliographic review of selected articles and major GI proceedings), were searched through January 2019. The primary outcome was the re-intervention rate. Secondary outcomes were a technical success, early and late complications, and stent malfunction rate. Pooled odds ratio (OR) and 95% confidence interval (CI) were calculated for each outcome.

RESULTS

A total of 9 studies were included (2 prospective Randomized Controlled Study, 5 retrospective studies, and 2 abstracts), involving 782 patients with malignant hilar obstruction. Bilateral stenting had significantly lower re-intervention rate compared with unilateral drainage (OR = 0.59, 95%CI: 0.40-0.87, P = 0.009). There was no difference in the technical success rate (OR = 0.7, CI: 0.42-1.17, P = 0.17), early complication rate (OR = 1.56, CI: 0.31-7.75, P = 0.59), late complication rate (OR = 0.91, CI: 0.58-1.41, P = 0.56) and stent malfunction (OR = 0.69, CI: 0.42-1.12, P = 0.14) between bilateral and unilateral stenting for malignant hilar biliary

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strictures.

CONCLUSION
Bilateral biliary drainage had a lower re-intervention rate as compared to unilateral drainage for high grade inoperable malignant biliary strictures, with no significant difference in technical success, and early or late complication rates.

Key words: Metal stent; Hilar biliary stricture; Re-intervention rate; Technical success rate

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Core tip: Biliary drainage is useful to control jaundice and cholangitis in patients with inoperable malignant hilar strictures. No consensus guidelines are available to decide if bilateral stenting has any advantage over unilateral stenting. This meta-analysis adds to the growing body of evidence that bilateral stenting is technically feasible with similar early and late complications and leads to lower re-intervention rates.

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INTRODUCTION

Unresectable malignant hilar obstruction (UMHO) is associated with very poor prognosis. Five-year survival is < 10% with most patients dying within 1 year of diagnosis[1,2]. Compared to plastic stents, self-expanding metallic stents (SEMS) have shown to be more cost effective and provides advantage with longer stent patency and less re-intervention rate in patients with non-operable malignancy with score of II to IV on Bismuth-Corlette classification and Hilar cholangiocarcinoma who have a predicted the life expectancy of > 3 mo[3-5]. Biliary stenting also plays a role in the management of obstructive jaundice and cholangitis and is important in enhancing the quality of life of patients with UMHO.

Although endoscopic stenting is widely favored in cases of UMHO, there is currently no consensus on whether the placement of bilateral biliary stents has any advantage for these patients over unilateral stenting. Although some experts believe in measuring the volume of the liver to be drained to determine the type of stent to be used, quantification of the liver volume is clinically challenging. Furthermore, there have been conflicting data regarding the technical success and outcomes of bilateral and unilateral stenting. While some authors believe that unilateral stenting renders increased technical success rate with concomitant lower complications[6-8]; bilateral stenting, on the other hand, will drain higher liver volume, may have longer stent patency, and hence may require less re-intervention[9-11].

The aim of the meta-analysis was to systematically review the current literature and compare the efficacy of unilateral *vs* bilateral stenting in achieving successful stent placement, comparing re-intervention rate, technical success, and early and late procedure-related complications for unresectable malignant hilar strictures.

MATERIALS AND METHODS

Data sources and searches

Search strategies were developed with the assistance of a health sciences librarian with expertise in searching for systematic reviews. Comprehensive search strategies using index and keywords were constructed for PubMed, Embase (Elsevier), and Cinhal (EBSCO). No database filters were used at any time during the searching process. All searches were conducted during January 2019 and the number of citations found in each database can be found in the flow diagram (Figure 1). The searches combined the following concepts: Unilateral SEMS and bilateral SEMS with biliary stents. Within the results for those combined concepts, additional filters, publication

types, and keyword strategies were used to identify and exclude the most common articles types that do not report trial results (reviews and case studies). An exhaustive forward search tool was used for the Web of Science database to capture all possible studies of interest. The databases were searched for publications dates 1995 to present. Language limits were applied to search for articles in English only. To identify further articles, references were hand searched. All results were downloaded into EndNote (Thompson ISI Research Soft, Philadelphia, PA, United States), a bibliographic database manager, and duplicate citations were identified and removed. In addition, abstracts from Digestive Disease Week, annual meetings of American College of Gastroenterology, and United European Gastroenterology Week from the last 5 years were also searched.

Inclusion criteria

Prospective studies, retrospective studies, and abstracts published in the English language were included if they compared unilateral *vs* bilateral SEMS biliary stent placement, for one or more of the clinical outcomes: Re-intervention rate, technical success, complication rate, and stent malfunction.

Exclusion criteria

Studies were excluded when there was no comparison between unilateral and bilateral stents. We also excluded studies that did not evaluate the required pre-defined endpoints. Furthermore, duplicate studies, case reports, animal studies, and letters to editors were excluded.

Data extraction

Two authors (Ashat M and Arora S) independently extracted the data according to a pre-specified protocol from all the included studies. All discrepancies were resolved after discussion with a third reviewer.

Quality assessment and risk of bias

Cohort studies were assessed using the Newcastle-Ottawa Scale and for randomized control trials, Cochrane tool was used to assess for risk of bias[12,13]. Risk of publication bias for each end-point was assessed using the funnel plots.

Outcome

The data collected from eligible studies included following data points-publication year, authors, country of publication, study design, mean age of study participants, a total number of patients in each unilateral stenting and bilateral stenting category and type of malignancy, complications rates, and type of complications Supplemental (Table 1).

Primary end-point of the study was the re-intervention rate. This was defined as an endoscopic or percutaneous intervention that was done for stent failure and to increase biliary drainage or for recurrent jaundice, or for management of dilated intra-hepatic bile duct revealed by imaging or management of immediate adverse event of successfully inserted SEMS. Secondary outcomes were (1) Technical success was defined by the successful placement of bilateral or unilateral SEMS across stricture site, confirmed by the flow of contrast or bile through SEMS; (2) Early adverse event rate- defined as early stent-related complications within 4 wk. Early complications included cholangitis, cholecystitis, pancreatitis, bleeding, and liver abscess; (3) Late adverse events were defined as any stent-related complication that occurred after 4 wk of stent insertion. Late complication included cholangitis, cholecystitis, liver abscess; and (4) Stent malfunction defined as stent obstruction due to sludge or stone formation, cholangitis, tumor in-growth, or development of a liver abscess, or biloma.

Statistical analysis

Review Manager 5.3 (The Cochrane Collaboration, Oxford, United Kingdom) was used to analyze the data for the meta-analysis. Pooled odds ratios (ORs) and 95% confidence interval (CI) of study end-points were calculated using the Mantel-Haenszel method. In order to access of heterogeneity, we used X^2 test (Cochran Q statistic). In case there was significant heterogeneity, a random-effect model was used. Funnel plots were obtained to assess the risk of bias.

RESULTS

Using pre-defined parameters and removing duplicate publications our search strategy identified 281 articles. Another 2 articles were identified by manual search. A total of 262 articles were excluded based on our exclusion criterion. Based on our

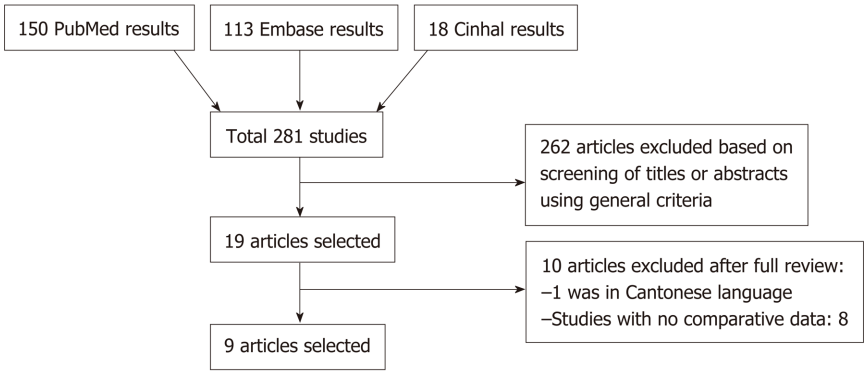

Figure 1 PRISMA diagram of the literature search.

inclusion criterions, 9 studies were selected (Figure 1). Of these 9 studies, 7 were published manuscripts and 2 were published as abstracts. All the baseline characteristics of each individual studies are highlighted in Supplemental Table 1.

Study characteristics

The characteristics of the studies, and of the patients in the selected studies are shown in Supplemental Table 1. A total of 9 studies were enrolled in the current study of which 2 were randomized control trial's (RCT), and 7 were retrospective trials (5 complete manuscripts and 2 abstracts). Although the study by Mukai *et al*[14] was an RCT, for our analysis we used only bilateral stents subgroup of the study which was not randomized. A total of 782 patients were included in the analysis of bilateral *vs* unilateral biliary stenting.

Results of meta-analysis

Primary end-point: Re-intervention rate: A total of 7 studies involving 513 patients was included in this analysis[7,10,14,15,17-20]. Bilateral stenting required significantly lower re-intervention as compared to unilateral stenting (OR = 0.59, 95%CI: 0.40-0.87, P = 0.009) (Figure 2). The funnel plot showed no asymmetry (Figure 3).

Secondary end-points: (1) Technical success: A total of 8 studies involving 745 patients was included in this analysis[7,10,14,15,17-20]. There was no significant difference in the technical success rate with bilateral stenting as compared to unilateral stenting (OR = 0.7, 95%CI: 0.42-1.17, P = 0.17) (Figure 4). There was mild heterogeneity; (2) Early complications: A total of 5 studies involving 530 patients were included in this analysis[7,10,18-20]. There was no difference between early complications (OR = 1.56, 95%CI: 0.31-7.75, P = 0.0001) (Figure 5); (3) Late complications: A total of 5 studies involving 430 patients were included in this analysis[7,10,15,18,20]. There was no difference in late complication rate (OR = 0.91, 95%CI: 0.58-1.41, P = 0.56) (Figure 6); and (4) Stent malfunction: A total of 4 studies involving 324 patients was included in this analysis[7,10,15,18]. There was no difference in stent malfunction rates (OR = 0.69, 95%CI: 0.42-1.12, P = 0.14) (Figure 7).

Quality assessment and funnel plots

The Newcastle Ottawa Scale score has been provided for all retrospective studies in Supplemental Table 1. The Cochrane collaboration tool assessment of bias for the RCT has been provided in Figure 8. Funnel plots to estimate bias revealed no asymmetry (Figure 3).

DISCUSSION

Endoscopic biliary drainage is the intervention of choice in patients with UMHO. Besides providing symptomatic relief to patients with pruritis it also has therapeutic implications with a reduction in total bilirubin which permits the use of subsequent chemotherapy, radiotherapy or photodynamic therapy. This may be important in prolonging the life of patients with unresectable malignant biliary strictures. Over the past decade, multiple studies have found using metallic stents over plastic stents as more cost-effective in hilar cholangiocarcinoma[4,14,21,22]. However, the data comparing bilateral *vs* unilateral stenting in UMHO is sparse. There has been conflicting data in

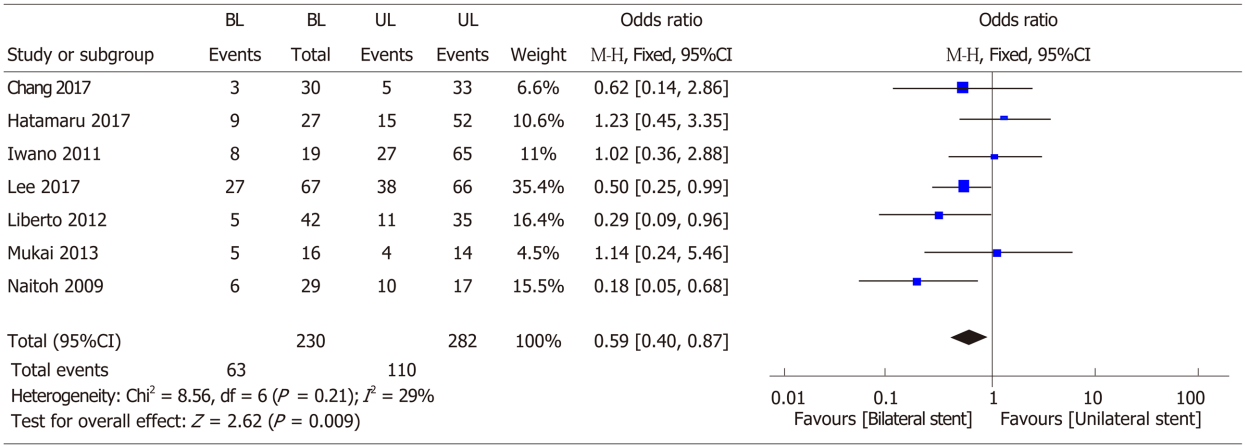

Figure 2 Forest plot of re-intervention rates with bilateral self expanding metal stents *vs* unilateral stent. SEMS: Self expanding metal stents; OR: Odds ratio; CI: Confidence interval.

regards to the outcomes of placement of bilateral *vs* unilateral SEMS stents[7,8,10,18,23,24]. Therefore, we designed this meta-analysis to review the data, thus-far available, comparing bilateral *vs* unilateral SEMS placement for UMHO. Our meta-analysis shows that bilateral stenting as compared to unilateral stenting is associated with a lower re-intervention rate, but has a comparable technical success rate, early and late complication rates.

Bilateral stenting was associated with a statistically significant 41% reduction in re-intervention rate. There has been marked variability in results among published literature. A prospective trial by Mukai *et al*[14] demonstrated 50% re-intervention rate in bilateral SEMS group compared to 29% in unilateral SEMS group. However, the study groups were not treated similarly in their study, as patients who received bilateral stent received sphincterotomy while patients receiving unilateral stent did not. In the prospective RCT by Lee *et al*[10], the authors showed a statistically significant lower re-intervention rate at 3 mo for bilateral SEMS group *vs* unilateral SEMS group (10.9% *vs* 33.3%). The ability to reduce the number of interventions is of paramount importance in patients with non-operable malignant hilar strictures and an average life expectancy < 12 mo, thus avoiding multiple hospitalizations, which in-return could mean an overall more cost-effective approach and also will have an impact on improving the quality of life for patients[18,25]. Further, restoration of bile flow with bilateral stenting is physiologically more superior to unilateral stenting. Approximately 25%-30% liver needs to be drained in order to satisfactorily reduce jaundice[8,26]. Though unilateral stent should be able to drain at-least 25% of the liver, clinical evidence suggests that up to 30% cases of hilar cholangiocarcinoma are associated with hepatic lobar atrophy[27], and thus in such a situation, unilateral stenting may not provide an appropriate therapeutic response and may increase primary re-intervention rates. Furthermore, a study by Vienne *et al*[28] suggested that draining more than 50% of the liver volume is an important predictor of the effectiveness of biliary drainage especially in malignant hilar strictures.

The conflicting data is further complicated by the technical difficulties associated with the placement of bilateral stents. Thus, multiple newer stent delivery systems have been developed to overcome this technical challenge. In our study, there was no significant difference in the technical success between bilateral biliary stenting and unilateral biliary stenting. A meta-analysis by Hong W *et al*[24] concluded higher success with unilateral stenting. However, their meta-analysis included studies involving plastic biliary stents which may have affected the results. Our results are similar to results by Naitoh *et al*[18] and Iwano *et al*[7] who had similar technical success for bilateral and unilateral stents. Bilateral stents could be placed by either stent-in-stent technique (SIS) or side-by-side technique (SBS). Naitoh *et al* use stent in stent technique and Iwano *et al* used SIS technique respectively in their patients Although, this meta-analysis did not specifically compare the two techniques of bilateral stenting, a meta-analysis by Naitoh *et al*[18] showed longer stent patency time with SBS group when compared to SIS group. Provider expertise could also account for the variability in the technical success rate among the studies. Thus, based on our results, we believe that bilateral stenting may be preferable for providers who are technically adept at placing both bilateral and unilateral stent.

Stent malfunction could be driving our primary outcome of stent re-intervention

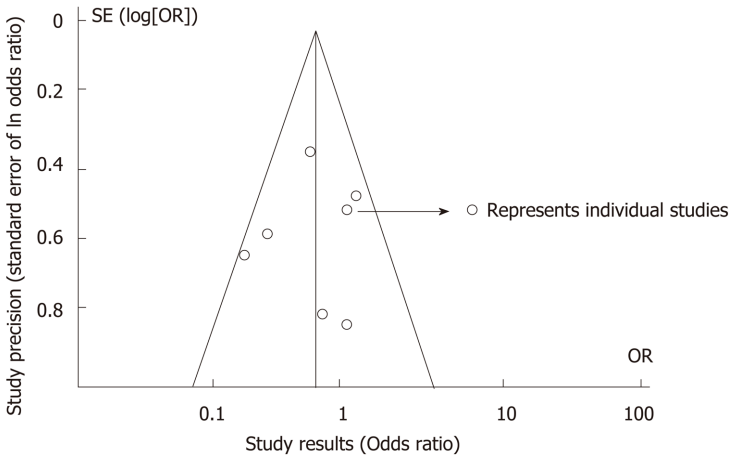

Figure 3 Cochrane collaboration tool. Risk assessment.

rates. The rate of reintervention was influenced by stent malfunction, however, not all studies defined the cause of stent malfunction clearly (Supplemental Table 2) and hence we only included stent malfunction as a secondary outcome. There was a trend towards decreased stent malfunction with bilateral drainage as compared to unilateral drainage though this was not statistically different. Earlier studies seemed to suggest that bilateral stents could lead to increased stent-related early complications. SBS was associated with increased cholangitis rates and portal vein occlusion because of excessive expansion of the bile duct by parallel stents[18]. SIS deployment could lead to increased sludge formation at the site of stent overlap as a result of a reduction in bile inflow and increase the incidence of tumor ingrowth if the stent mesh is expanded in the area of overlap[17]. In contrast, most recent RCT by Lee *et al*[10] has shown no difference in rates of cholangitis and liver abscess after bilateral stent placement. Similarly, in our meta-analysis, there was no difference with stent-related early or late complication rates between the two groups.

There are several limitations to this meta-analysis. The main limitation is that only two studies included in our meta-analysis are RCT's. Most studies are retrospective studies which could have led to selection bias. Nevertheless, the retrospective studies are reasonable quality cohort studies, as determined by the Modified Newcastle Ottawa quality assessment scale of cohort studies. Another limitation is the presence of significant heterogeneity in some of the analysis. This is likely due to the significant clinical heterogeneity among the studies the differences in the study population, the location of malignant strictures, technical expertise of the providers, and the difference in the duration. However, importantly, there was only mild heterogeneity in the analysis of our primary end-point analysis of re-intervention rate and in the analysis for technical success. For analysis with significant heterogeneity, we used a random effects model to partly account for the clinical heterogeneity. This highlights the need for further research on this topic and the importance of our meta-analysis based on available data.

In conclusion, bilateral biliary stenting for UMHO may decrease the re-intervention rate in patients with malignant hilar strictures, without increasing early or late complication rate. To the best of our knowledge, this is the first meta-analysis so far comparing the outcomes of SEMS bilateral *vs* unilateral stenting. Further RCT's are needed to confirm our findings.

Study or subgroup	BL Events	BL Total	UL Events	UL Events	Weight	Odds ratio M-H, Fixed, 95%CI
Chang 2017	27	30	31	33	8.3%	0.58 [0.09, 3.74]
Cheng 2017	60	83	53	80	42.2%	1.33 [0.68, 2.59]
Iwano 2011	17	19	62	65	8.3%	0.41 [0.06, 2.66]
Lee 2017	64	67	66	66	9.7%	0.14 [0.01, 2.74]
Liberto 2012	42	45	35	35	8.6%	0.17 [0.01, 3.42]
Mukai 2013	16	16	14	14		Not estimable
Naitoh 2009	26	29	17	17	7.2%	0.22 [0.01, 4.45]
Teng 2019	47	52	54	54	15.7%	0.08 [0.00, 1.47]
Total (95%CI)		341		364	100%	0.70 [0.42, 1.17]
Total events	299		332			

Heterogeneity: Chi^2 = 8.59, df = 6 (P = 0.20); I^2 = 30%
Test for overall effect: Z = 1.37 (P = 0.17)

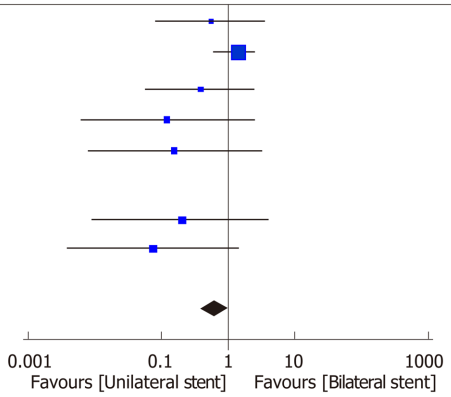

Figure 4 Forest plot of technical success rates with bilateral self expanding metal stents *vs* unilateral stent. SEMS: Self expanding metal stents; OR: Odds ratio; CI: Confidence interval.

Study or subgroup	BL Events	BL Total	UL Events	UL Events	Weight	Odds ratio M-H, Fixed, 95%CI
Cheng 2017	39	83	23	80	26.6%	2.20 [1.15, 4.20]
Iwano 2011	4	17	0	65	14.2%	43.67 [2.22, 859.61]
Lee 2017	5	67	21	66	24.8%	0.17 [0.06, 0.49]
Naitoh 2009	3	29	0	17	14%	4.62 [0.22, 95.11]
Teng 2019	2	52	3	54	20.4%	0.68 [0.11, 4.24]
Total (95%CI)		248		282	100%	1.56 [0.31, 7.75]
Total events	53		47			

Heterogeneity: Tau^2 = 2.41, Chi^2 = 23.20 df = 4 (P = 0.0001); I^2 = 83%
Test for overall effect: Z = 0.54 (P = 0.59)

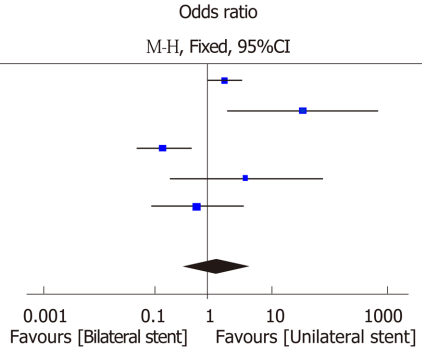

Figure 5 Forest plot of early complication rates with bilateral self expanding metal stents *vs* unilateral stent. SEMS: Self expanding metal stents; OR: Odds ratio; CI: Confidence interval.

Study or subgroup	BL Events	BL Total	UL Events	UL Events	Weight	Odds ratio M-H, Fixed, 95%CI
Chang 2017	7	30	4	33	7%	2.21 [0.57, 8.47]
Iwano 2011	14	19	45	65	12.9%	1.24 [0.39, 3.93]
Lee 2017	34	67	37	66	44.3%	0.81 [0.41, 1.60]
Naitoh 2009	14	29	11	17	17.3%	0.51 [0.15, 1.75]
Teng 2019	7	52	9	54	18.4%	0.78 [0.27, 2.27]
Total (95%CI)		197		235	100%	0.91 [0.58, 1.41]
Total events	76		106			

Heterogeneity: Chi^2 = 3.00 df = 4 (P = 0.56); I^2 = 0%
Test for overall effect: Z = 0.44 (P = 0.66)

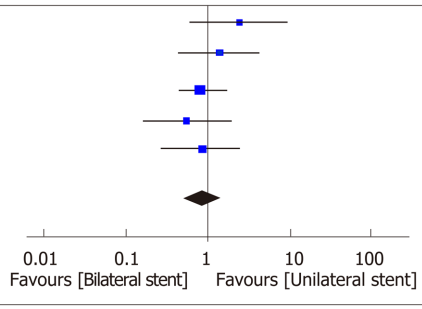

Figure 6 Forest plot of late complication rates with bilateral self expanding metal stents *vs* unilateral stent. SEMS: Self expanding metal stents; OR: Odds ratio; CI: Confidence interval.

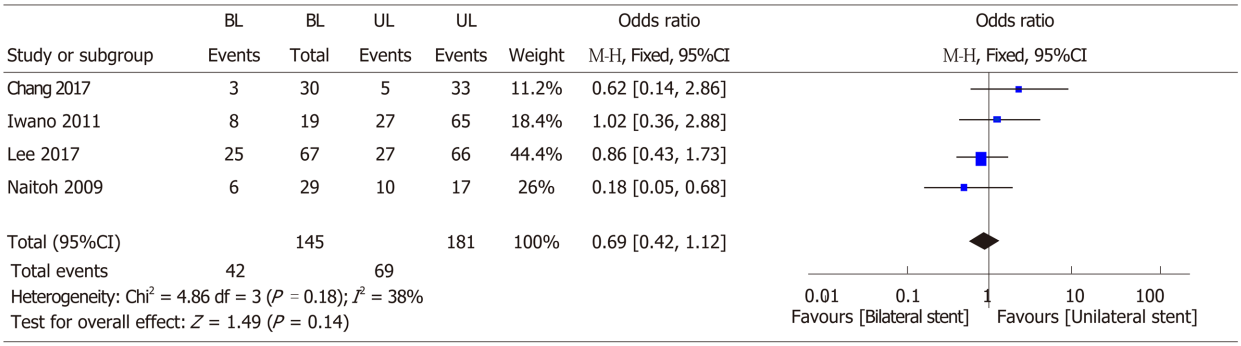

Figure 7 Forest plot of stent malfunction rates with bilateral self expanding metal stents *vs* unilateral stent. SEMS: Self expanding metal stents; OR: Odds ratio; CI: Confidence interval.

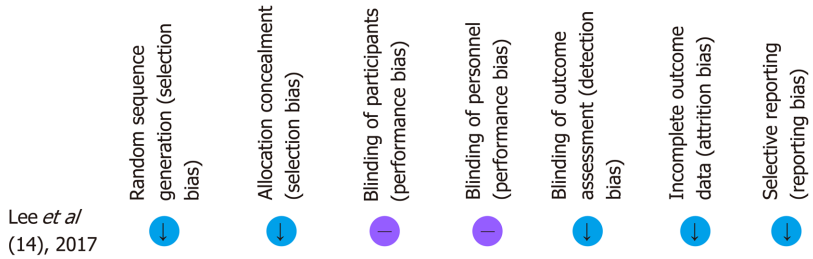

Figure 8 Risk of bias summary: Review authors' judgements about each risk of bias item for each included study.

ARTICLE HIGHLIGHTS

Research background

Inoperable malignant biliary strictures comes with a very high mortality rate. Self expanding metal stents (SEMS) not only offers symptomatic relief but also gives an opportunity for these patients to receive chemotherapy once bilirubin starts to trend down. Unilateral biliary SEMS have long been thought to be adequate and probably better than bilateral SEMS as it comes with lower complication rates. However, with newer endoscopic techniques and most recent prospective trials, the efficacy of bilateral SEMS has shown to be better than unilateral SEMS and with similar complication rates. This meta-analysis highlights the growing body of evidence in support of bilateral stenting versus unilateral stenting.

Research motivation

Over the past few years, newer randomized control trials (RCTs have been published showing the overall advantage of bilateral biliary stenting over unilateral stenting in a subset of patients with inoperable hilar malignant strictures. No meta-analysis was done on this topic with newer study data points.

Research objectives

We aimed to conduct a meta-analysis to compare the role of bilateral stenting *vs* unilateral stenting in inoperable malignant hilar strictures.

Research methods

A detailed literature search was conducted to find all the relevant articles. Two reviewers independently analyzed all the selected studies. All discrepancies were discussed independently with the third reviewer and consensus was achieved. We used Pooled odds ratio (OR) and 95% confidence intervals (CIs) were calculated for each outcome.

Research results

A total of 782 patients form nine studies were included for analysis. Bilateral stenting had significantly lower re-intervention rate compared with unilateral drainage (OR = 0.59, 95%CI: 0.40-0.87, P = 0.009). There was no difference in the technical success rate (OR = 0.7, CI: 0.42-1.17, P = 0.17), early complication rate (OR = 1.56, CI: 0.31-7.75, P = 0.59), late complication rate (OR = 0.91, CI: 0.58-1.41, P = 0.56) and stent malfunction (OR = 0.69, CI: 0.42-1.12, P = 0.14) between bilateral and unilateral stenting for malignant hilar biliary strictures.

Research conclusions

Older studies that have shown the ease of putting unilateral stenting with fewer complications

over bilateral stenting in inoperable malignant hilar strictures. However, with new RCTs showing the higher success of bilateral biliary stenting with lower re-intervention rates, bilateral stenting could offer an overall advantage over unilateral stenting. Our study highlights the overall advantage of bilateral stenting over unilateral stenting.

Research perspectives

Biliary stenting is very important modality in the overall management of inoperable malignant hilar strictures. Bilateral stenting offers an advantage over unilateral stenting, however more RCT is required to further support this conclusion.

REFERENCES

1 **Bismuth H**, Castaing D, Traynor O. Resection or palliation: priority of surgery in the treatment of hilar cancer. *World J Surg* 1988; **12**: 39-47 [PMID: 2449769 DOI: 10.1007/BF01658484]

2 **Black K**, Hanna SS, Langer B, Jirsch DW, Rider WD. Management of carcinoma of the extrahepatic bile ducts. *Can J Surg* 1978; **21**: 542-545 [PMID: 83901]

3 **Rerknimitr R**, Angsuwatcharakon P, Ratanachu-ek T, Khor CJ, Ponnudurai R, Moon JH, Seo DW, Pantongrag-Brown L, Sangchan A, Pisespongsa P, Akaraviputh T, Reddy ND, Maydeo A, Itoi T, Pausawasdi N, Punamiya S, Attasaranya S, Devereaux B, Ramchandani M, Goh KL; Asia-Pacific Working Group on Hepatobiliary Cancers. Asia-Pacific consensus recommendations for endoscopic and interventional management of hilar cholangiocarcinoma. *J Gastroenterol Hepatol* 2013; **28**: 593-607 [PMID: 23350673 DOI: 10.1111/jgh.12128]

4 **Dumonceau JM**, Tringali A, Papanikolaou IS, Blero D, Mangiavillano B, Schmidt A, Vanbiervliet G, Costamagna G, Devière J, García-Cano J, Gyökeres T, Hassan C, Prat F, Siersema PD, van Hooft JE. Endoscopic biliary stenting: indications, choice of stents, and results: European Society of Gastrointestinal Endoscopy (ESGE) Clinical Guideline - Updated October 2017. *Endoscopy* 2018; **50**: 910-930 [PMID: 30086596 DOI: 10.1055/a-0659-9864]

5 **Dumonceau JM**, Tringali A, Blero D, Devière J, Laugiers R, Heresbach D, Costamagna G; European Society of Gastrointestinal Endoscopy. Biliary stenting: indications, choice of stents and results: European Society of Gastrointestinal Endoscopy (ESGE) clinical guideline. *Endoscopy* 2012; **44**: 277-298 [PMID: 22297801 DOI: 10.1055/s-0031-1291633]

6 **Polydorou AA**, Chisholm EM, Romanos AA, Dowsett JF, Cotton PB, Hatfield AR, Russell RC. A comparison of right *vs* left hepatic duct endoprosthesis insertion in malignant hilar biliary obstruction. *Endoscopy* 1989; **21**: 266-271 [PMID: 2482169 DOI: 10.1055/s-2007-1012966]

7 **Iwano H**, Ryozawa S, Ishigaki N, Taba K, Senyo M, Yoshida K, Sakaida I. Unilateral *vs* bilateral drainage using self-expandable metallic stent for unresectable hilar biliary obstruction. *Dig Endosc* 2011; **23**: 43-48 [PMID: 21198916 DOI: 10.1111/j.1443-1661.2010.01036.x]

8 **De Palma GD**, Galloro G, Siciliano S, Iovino P, Catanzano C. Unilateral *vs* bilateral endoscopic hepatic duct drainage in patients with malignant hilar biliary obstruction: results of a prospective, randomized, and controlled study. *Gastrointest Endosc* 2001; **53**: 547-553 [PMID: 11323577 DOI: 10.1067/mge.2001.113381]

9 **Kogure H**, Isayama H, Kawakubo K, Sasaki T, Yamamoto N, Hirano K, Sasahira N, Tsujino T, Tada M, Koike K. Endoscopic bilateral metallic stenting for malignant hilar obstruction using newly designed stents. *Journal of Hepato-Biliary-Pancreatic Sciences* 2011; **18**: 653-657 [PMID: 21681649 DOI: 10.1007/s00534-011-0407-4]

10 **Lee TH**, Kim TH, Moon JH, Lee SH, Choi HJ, Hwangbo Y, Hyun JJ, Choi JH, Jeong S, Kim JH, Park DH, Han JH, Park SH. Bilateral *vs* unilateral placement of metal stents for inoperable high-grade malignant hilar biliary strictures: a multicenter, prospective, randomized study (with video). *Gastrointest Endosc* 2017; **86**: 817-827 [PMID: 28479493 DOI: 10.1016/j.gie.2017.04.037]

11 **Kogure H**, Isayama H, Nakai Y, Tsujino T, Matsubara S, Yashima Y, Ito Y, Hamada T, Takahara N, Miyabayashi K, Mizuno S, Mohri D, Kawakubo K, Sasaki T, Yamamoto N, Hirano K, Sasahira N, Tada M, Koike K. High single-session success rate of endoscopic bilateral stent-in-stent placement with modified large cell Niti-S stents for malignant hilar biliary obstruction. *Dig Endos* 2014; **26**: 93-99 [PMID: 23517109 DOI: 10.1111/den.12055]

12 **Higgins JPT**, Altman DG, Gøtzsche PC, Jüni P, Moher D, Oxman AD, Savović J, Schulz KF, Weeks L, Sterne JAC. The Cochrane Collaboration's tool for assessing risk of bias in randomised trials. *BMJ* 2011; **343**: d5928 [PMID: 22008217 DOI: 10.1136/bmj.d5928]

13 **Stang A**; Critical evaluation of the Newcastle-Ottawa scale for the assessment of the quality of nonrandomized studies in meta-analyses. *Eur J Epidemiol* 2010; **25**: 603-605 [PMID: 20652370 DOI: 10.1007/s10654-010-9491-z]

14 **Mukai T**, Yasuda I, Nakashima M, Doi S, Iwashita T, Iwata K, Kato T, Tomita E, Moriwaki H. Metallic stents are more efficacious than plastic stents in unresectable malignant hilar biliary strictures: a randomized controlled trial. *J Hepatobiliary Pancreat Sci* 2013; **20**: 214-222 [PMID: 22415652 DOI: 10.1007/s00534-012-0508-8]

15 **Chang G**, Xia FF, Li HF, Niu S, Xu YS. Unilateral *vs* bilateral stent insertion for malignant hilar biliary obstruction. *Abdom Radiol (NY)* 2017; **42**: 2745-2751 [PMID: 28477177 DOI: 10.1007/s00261-017-1174-8]

16 **Hatamaru K**, Yamashita Y, Uenoyama Y. Tu1448 The Study of Endoscopic Treatment for Unresectable Malignant Hilar Biliary Obstruction. *Gastrointest Endosc* 2017; **85**: AB626 [DOI: 10.1016/j.gie.2017.03.1455]

17 **Liberato MJ**, Canena JM. Endoscopic stenting for hilar cholangiocarcinoma: efficacy of unilateral and bilateral placement of plastic and metal stents in a retrospective review of 480 patients. *BMC Gastroenterol* 2012; **12**: 103 [PMID: 22873816 DOI: 10.1186/1471-230x-12-103]

18 **Naitoh I**, Ohara H, Nakazawa T, Ando T, Hayashi K, Okumura F, Okayama Y, Sano H, Kitajima Y, Hirai M, Ban T, Miyabe K, Ueno K, Yamashita H, Joh T. Unilateral *vs* bilateral endoscopic metal stenting for malignant hilar biliary obstruction. *J Gastroenterol Hepatol* 2009; **24**: 552-557 [PMID: 19220678 DOI: 10.1111/j.1440-1746.2008.05750.x]

19 **Cheng S**, Jianfeng y, Zhang X. Tu1408 A Comparison of Unilateral *Vs* Bilateral Endoscopic Biliary

Stenting for Bismuth-Corlette II-IV Malignant Hilar Strictures. *Gastrointest Endosc* 2017; **85**: AB613 [DOI: 10.1016/j.gie.2017.03.1415]
20 **Teng F**, Xian YT, Lin J, Li Y, Wu AL. Comparison of Unilateral With Bilateral Metal Stenting for Malignant Hilar Biliary Obstruction. *Surg Laparosc Endosc Percutan Tech* 2019; **29**: 43-48 [PMID: 30418421 DOI: 10.1097/SLE.0000000000000594]
21 **Perdue DG**, Freeman ML, DiSario JA, Nelson DB, Fennerty MB, Lee JG, Overby CS, Ryan ME, Bochna GS, Snady HW, Moore JP; ERCP Outcome Study ERCOST Group. Plastic *vs* self-expanding metallic stents for malignant hilar biliary obstruction: a prospective multicenter observational cohort study. *J Clin Gastroenterol* 2008; **42**: 1040-1046 [PMID: 18719507 DOI: 10.1097/MCG.0b013e31815853e0]
22 **Sangchan A**, Kongkasame W, Pugkhem A, Jenwitheesuk K, Mairiang P. Efficacy of metal and plastic stents in unresectable complex hilar cholangiocarcinoma: a randomized controlled trial. *Gastrointest Endosc* 2012; **76**: 93-99 [PMID: 22595446 DOI: 10.1016/j.gie.2012.02.048]
23 **De Palma GD**, Pezzullo A, Rega M, Persico M, Patrone F, Mastantuono L, Persico G. Unilateral placement of metallic stents for malignant hilar obstruction: a prospective study. *Gastrointest Endosc* 2003; **58**: 50-53 [PMID: 12838220 DOI: 10.1067/mge.2003.310]
24 **Hong W**, Sun X, Zhu Q. Endoscopic stenting for malignant hilar biliary obstruction: should it be metal or plastic and unilateral or bilateral? *Eur J Gastroenterol Hepatol* 2013; **25**: 1105-1112 [PMID: 23542449 DOI: 10.1097/MEG.0b013e328360b9ec]
25 **Abraham NS**, Barkun JS, Barkun AN. Palliation of malignant biliary obstruction: a prospective trial examining impact on quality of life. *Gastrointest Endosc* 2002; **56**: 835-841 [PMID: 12447294 DOI: 10.1067/mge.2002.129868]
26 **Larghi A**, Tringali A, Lecca PG, Giordano M, Costamagna G. Management of hilar biliary strictures. *Am J Gastroenterol* 2008; **103**: 458-473 [PMID: 18028506 DOI: 10.1111/j.1572-0241.2007.01645.x]
27 **Gwon DI**, Ko GY, Sung KB, Yoon HK, Shin JH, Hyoung Kim J, Kim J, Oh JY. Percutaneous biliary metallic stent placement in patients with unilobar portal vein occlusion caused by advanced hilar malignancy: outcome of unilateral *vs* bilateral stenting. *AJR Am J Roentgenol* 2011; **197**: 795-801 [PMID: 21940566 DOI: 10.2214/AJR.11.6424]
28 **Vienne A**, Hobeika E, Gouya H, Lapidus N, Fritsch J, Choury AD, Chryssostalis A, Gaudric M, Pelletier G, Buffet C, Chaussade S, Prat F. Prediction of drainage effectiveness during endoscopic stenting of malignant hilar strictures: the role of liver volume assessment. *Gastrointest Endosc* 2010; **72**: 728-735 [PMID: 20883850 DOI: 10.1016/j.gie.2010.06.040]

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