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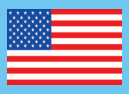

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Taiyuan Research and Treatment Center for Digestive Diseases, 77 Shuangta Xijie, Taiyuan 030001, Shanxi Province, China

EDITING
Editorial Board of *World Journal of Gastroenterology*
Room 903, Building D, Ocean International Center,
No. 62 Dongsihuan Zhonglu, Chaoyang District,
Beijing 100025, China
Telephone: +86-10-59080039
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E-mail: wjg@wjgnet.com
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EDITORIAL OFFICE
Jian-Xia Cheng, Director
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World Journal of Gastroenterology
Room 903, Building D, Ocean International Center,
No. 62 Dongsihuan Zhonglu, Chaoyang District,
Beijing 100025, China
Telephone: +86-10-59080039
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com
http://www.wjgnet.com

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EDITORIAL

Role of taxanes in pancreatic cancer

Carmen Belli, Stefano Cereda, Michele Reni

Carmen Belli, Stefano Cereda, Michele Reni, Department of Oncology, San Raffaele Scientific Institute, 20132 Milan, Italy
Author contributions: Reni M conceived the paper and critically reviewed the data and the final version; Cereda S contributed to the analysis and interpretation of data; Belli C drafted the article and revised it critically for important intellectual content; and all authors approved the final version.
Correspondence to: Michele Reni, MD, Department of Oncology, San Raffaele Scientific Institute, via Olgettina 60, 20132 Milan, Italy. reni.michele@hsr.it
Telephone: +39-2-26437644 Fax: +39-2-26437625
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Abstract

Pancreatic cancer is one of the most deadly cancers and is characterized by a poor prognosis. Single agent gemcitabine, despite its limited activity and modest impact on disease outcome, is considered as the standard therapy in pancreatic cancer. Most of the combination regimens used in the treatment of this disease, also including the targeted agents, did not improve the outcome of patients. Also, taxanes have been tested as single agent and in combination chemotherapy, both in first line and as salvage chemotherapy, as another possible option for treating pancreatic cancer. The inclusion of taxanes in combination with gemcitabine as upfront therapy obtained promising results. Accordingly, taxanes, and above all, new generation taxanes, appear to be suitable candidates for further testing to assess their role against pancreatic cancer in various clinical settings.

Key words: Pancreatic cancer; Advanced disease; Metastatic disease; Chemotherapy; Taxanes; Drug combinations; Radiotherapy; ABI-007

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INTRODUCTION

Pancreatic cancer has a very poor prognosis and it still remains as one of the most deadly cancers. At diagnosis, only 10%-20% of patients are considered candidates for a curative resection that is possible only in the absence of distant metastases, peritoneal carcinomatosis, and lack of any involvement of celiac axis and superior mesenteric artery[1]. Around 80% of patients have advanced or metastatic disease and median overall survival (mOS) of this group is very poor ranging from 3 to 6 mo[2]. The first drug used in the treatment of advanced pancreatic cancer was 5-fluorouracil (5-FU) that provided a palliative benefit with a significant improvement in mOS compared to best supportive care (6 mo *vs* 2.5 mo, $P < 0.01$)[3]. When compared to bolus 5-FU, gemcitabine yielded a significantly better response rate (RR) (5.4% *vs* 0%), survival (mOS 5.65 mo *vs* 4.41 mo, $P = 0.0025$) and clinical benefit, which is a composite measure consisting of reduction of pain, analgesic drugs intake and weight loss (23.8% *vs* 4.8%)[4]. Given the modest and disappointing impact on overall survival (OS) achieved with single agent gemcitabine, other agents or drug combinations are continuously tested in advanced pancreatic cancer[5-7]. Among others, taxanes (docetaxel and pacli-

taxel) were tested as single agents or in combination with other chemotherapeutic agents in pancreatic cancer[8-10] because they showed promising activity in other solid tumours[11,12]. Mechanism of action of these drugs consists in enhancing microtubule assembly and inhibiting the depolymerization of tubulin responsible for the formation of bundles of microtubules blocking cell proliferation[13].

This article summarises the main clinical studies conducted in pancreatic cancer with taxanes as first or second line chemotherapy, both as monotherapy and combination therapy, in order to clarify the potential role of this class of drugs for further investigations.

DOCETAXEL

First-line therapy

Single agent: Docetaxel gained researchers' attention for the treatment of pancreatic cancer after a preclinical study showed its effectiveness in a murine model of pancreatic ductal adenocarcinoma[14]. Docetaxel activity as single agent was assessed in phase Ⅱ trials in chemonaïve patients affected by pancreatic cancer at two different dosages, 60 mg/mq[8] and 100 mg/mq[9,15], showing promising activity when used at higher dose (Table 1). With higher doses[9,15], the overall RR ranged from 5% to 15%, the median time to progression (mTTP) was 2.1-5.0 mo, and the mOS was 7-8.5 mo. Neutropenia was the most frequently observed grade 3/4 toxicity in both studies (36%-95%)[9]. Grade 3-4 anemia (9%-16%) and fatigue (9%-23%) were also commonly reported[9]. Given the phase Ⅱ nature of the trials, the small sample size and the selection of patient population, including stage Ⅱ and Ⅲ disease and patients who mainly had a performance status of 0-1, these results should be interpreted with caution. Nevertheless, these trials showed that docetaxel has some activity in the treatment of pancreatic cancer, and warrant further exploration. Docetaxel administered at lower dose failed to demonstrate any activity in pancreatic cancer[8]. In fact, no objective response was observed and both mTTP and mOS were shorter when compared to higher doses[9,15]. Moreover, grade 3-4 toxicity was remarkable with nearly 80% of the patients developing neutropenia, 7% anaemia, and 27% fatigue[8].

Combination chemotherapy: Docetaxel role was also addressed in combination with other drugs (Table 1). *In vitro* and *in vivo* studies have demonstrated that docetaxel yields a synergism with other drugs like capecitabine[16,17], 5-FU[18], gemcitabine[19,20], and cisplatin[21] .

The rationale for the combination of an oral fluoropyrimidine with docetaxel is based on the ability of taxanes to increase the activity in the tumoral tissue of thymidylate phosphorylases, which are key enzymes in the transformation process of capecitabine into its active metabolite 5-FU. The synergism of docetaxel with gemcitabine was observed *in vitro* in several cancer cell lines, but the biological mechanism is not clear. Hypothetically, the combination of these two agents could regulate the apoptotic process by increasing the apoptotic index. Finally, the synergism of docetaxel and cisplatin, observed in cell lines of gastric cancer, could be due to the down-regulation of multi drug resistant proteins by docetaxel thereby increasing cytotoxic index of cisplatin[21].

Several phase Ⅱ clinical trials assessed the activity of these combinations against advanced pancreatic cancer as an upfront therapy[19,20,22-26]. Docetaxel and gemcitabine yielded promising RR ranging from 12% to 40% and mOS ranging from 6 to 9 mo[19,20,23,25]. However, these single arm findings cannot be considered conclusive. Interestingly, the European Organization for Research and Treatment of Cancer (EORTC) group conducted a phase Ⅱ trial in which 96 patients were randomized to receive gemcitabine plus docetaxel or cisplatin plus docetaxel[24]. In 70 patients who were assessable for response, the RR was 19.4% with gemcitabine-docetaxel combination and 23.5% with cisplatin-docetaxel combination. Conversely, survival figures were better in 49 patients treated by the gemcitabine-docetaxel combination [median progression free survival (mPFS) 3.9 mo, mOS 7.4 mo; 1-year OS 30%] compared to those receiving cisplatin-docetaxel (mPFS 2.8 mo, mOS 7.1 mo; 1-year OS 16%). Toxicity was not negligible, consisting of grade 3-4 neutropenia in 47% and 55%, and febrile neutropenia in 9% and 16%, respectively. Altogether, safety profile and survival analysis favoured gemcitabine-docetaxel combination for further evaluation. Another phase Ⅱ trial randomized 259 patients with metastatic pancreatic cancer to receive fixed dose gemcitabine or gemcitabine combined with either docetaxel, cisplatin, or irinotecan[27]. The primary end point of this study was the six months OS that was similar in all four treatment arms: 57% for fixed gemcitabine dose, 53% for gemcitabine plus cisplatin, 54% for gemcitabine plus docetaxel, and 57% for gemcitabine plus irinotecan. The mOS and mTTP were similar in the treatment groups and ranged between 6.4-7.1 mo and 3.3-4.5 mo, respectively. The RRs were also indistinguishable among treatment groups and ranged between 12% to 14%. The cisplatin and docetaxel combination tested in this study gave similar results, in terms of mOS and TTP, to that reported in the EORTC trial[24].

Another drug tested in combination with docetaxel was liposomal doxorubicin starting from preclinical study conducted on xenografted human pancreatic carcinoma in which this drug demonstrated to reduce tumor growth with a low toxicity[28]. In a phase Ⅱ clinical trial, this combination was studied on twenty-one locally advanced and metastatic pancreatic cancer patients[29]. The results in terms of RR (21%) and mOS (10 mo) were similar to those observed with combination of docetaxel with other drugs[19,20,22-26].

The activity of docetaxel, gemcitabine, capecitabine regimen (GTX) was also tested in 43 patients with metastatic disease yielding a RR of 22% and a mOS of 14.5 mo[30]. These results were echoed in a recent retrospective study on 79 chemonaïve patients with locally advanced or metastatic pancreatic cancer who had a mOS

Table 1 Clinical trials of docetaxel in pancreatic cancer

Trial	CT agent	Line CT	No. of patients	RR %	mPFS (mo)	mOS (mo)	Toxicity %
Okada *et al*[8]	Doce	I	21	CR 0 PR 0 SD 33	1[1]	6	Neutropenia 86, anemia 10, thrombocytopenia 5, asthenia 33, nausea-vomiting 29
Androulakis *et al*[9]	Doce	I	33	CR 3 PR 3 SD 58	5[1]	8.5	Neutropenia 36, febrile neutropenia 6, anemia 9, asthenia 9, neuropathy 6
Rougier *et al*[15]	Doce	I	43	CR 0 PR 15 SD 3	2.1[1]	7	Neutropenia 95, febrile neutropenia 9, anemia 16, asthenia 23, vomiting 7
Stathopoulos *et al*[23]	Doce + GEM	I	54	CR 0 PR 13 SD 33	8[1]	6	Neutropenia 31, febrile neutropenia 11, thrombocytopenia 7, asthenia 13, diarrhea 6
Ryan *et al*[25]	Doce + GEM	I	33	CR 0 PR 18 SD 39	3.8	8.9	Neutropenia 49, febrile neutropenia 12, asthenia 27, nausea-vomiting 12, diarrhea 12, neuropathy 9
Lutz *et al*[24]	Doce + GEM Doce + CDDP	I	96	CR 0/2.9 PR 19.4/20.6 SD 36.1/35.3	3.9/2.8	7.4/7.1	Neutropenia 40/50, febrile neutropenia 9/16, anemia 20/9, thrombocytopenia 8/5, diarrhea 8/5, stomatitis 8/10
Kulke *et al*[27]	GEM + CDDP GEM FDR GEM + Doce GEM + CPT-11	I	259	CR 2/0/0/2 PR 11/14/12/12 SD 54/58/53/55	4.5/3.3/4.1/4.0	6.7/6.4/6.4/7.1	Neutropenia 46/48/31/25, febrile neutropenia 2/3/5/2, anemia 16/12/16/5, thrombocytopenia 49/25/9/14, asthenia 16/14/21/19, nausea-vomiting 41/26/17/25, diarrhea 80/2/8/8
Fine *et al*[30]	GTX	I	43	CR 0 PR 21.9 SD 41.5	6.9[1]	14.5	Neutropenia 29.2, thrombocytopenia 12.2, mucositis 7.5
Reni *et al*[22]	PDXG PEXG	I	105	CR 2/4 PR 58/33 SD 19/46	7.4/7.6	10.7/11	Neutropenia 4/13, thrombocytopenia 2/4, anemia 4/4, asthenia 6/3
Cereda *et al*[35]	Doce	II	10	CR 0 PR 0 SD 20	1.5	4	Not observed
Katopodis *et al*[37]	Doce + X	II	31	CR 0 PR 9.7 SD 22.6	2.4	6.3	Neutropenia 32.2, febrile neutropenia 3.2, anemia 3.2, thrombocytopenia 3.2, stomatitis 3.2, asthenia 6.5
Reni *et al*[39]	MDI	II-III	15	CR 0 PR 0 SD 20	1.7	6.1	Phase I study Neutropenia 23, fatigue, diarrhea, and vomiting 10

[1]mTTP: Median time to progression; CT: Chemotherapy; RR: Response rate; mPFS: Median progression free survival; mOS: Median overall survival; Doce: Docetaxel; GEM: Gemcitabine; CDDP: Cisplatin; CPT-11: Irinotecan; GTX: Gemcitabine + Taxotere + Xeloda; PDXG: Cisplatin + Docetaxel + Gemcitabine + Xeloda; PEXG: Cisplatin + Epirubicin + Xeloda + Gemcitabine; X: Xeloda; MDI: Mitomycin + Docetaxel + Irinotecan; CR: Complete response; PR: Partial response; SD: Stable disease.

of 25.0 and 11.3 mo, respectively[31].

A four drug combination of cisplatin, docetaxel, capecitabine, and gemcitabine (PDXG) was tested in a randomized phase II trial in which a cisplatin, epirubicin, capecitabine, and gemcitabine (PEXG) regimen was chosen as calibration arm[22]. This choice was based on the fact that a PEFG regimen (cisplatin, epirubicin, fluorouracil, and gemcitabine) was previously shown to be superior to gemcitabine monochemotherapy in terms of progression free survival [PFS; hazard ratio (HR) 0.51; range 0.33-0.78] and OS (HR 0.65; range 0.43-0.99) in a phase III trial of first line therapy of pancreatic cancer[32] and that the use of oral capecitabine was shown to be equivalent to 5-FU in other tumors[33]. Both the radiological and the biochemical RR[34] were better for 53 patients treated with PDXG (60% complete plus partial radiological responses; 41% major biochemical responses; 39% minor biochemical responses) than for 52 patients receiving PEXG (37% complete plus partial radiological responses; 32% major biochemical responses; 32% minor biochemical responses). However, OS and PFS were very similar in the two arms (mOS 10.7 mo *vs* 11.0 mo and mPFS 7.4 mo *vs* 7.6 mo, with PDXG and PEXG regimens, respectively). The safety profile of PDXG regimen was more favourable than that of PEXG regimen in terms of grade 3-4 neutropenia (4% in PDXG group *vs* 13% in PEXG arm).

Overall, these studies suggest that multi-drug associations, in particular triplets and quadruplets, are more active in pancreatic cancer when compared to monochemotherapy.

Salvage therapy

Single agent and combination chemotherapy: Docetaxel was also tested as salvage treatment in pancreatic cancer both as single agent and in combination[31,35-40]

Table 2 Clinical trials of paclitaxel as single agent or in combination chemotherapy in pancreatic cancer

Trial	CT agent	Line CT	No. of patients	RR %	mPFS (mo)	mOS (mo)	Toxicity %
Whitehead *et al*[10]	PTX	I	45	CR 3 PR 5 SD 13	NR	5	Neutropenia + leukopenia 92 , anemia 23, thrombocytopenia 20, asthenia 23, nausea-vomiting 18, neuropathy 7
Saif *et al*[46]	GPM	I	56	CR 1.7 PR 3.5 SD 54.8	2.8	6.5	Neutropenia 40, asthenia 17.8, neuropathy 13.3
Löhr *et al*[48]	GEM GEM + ET 11 mg/mq GEM + ET 22 mg/mq GEM + ET 44 mg/mq	I	212	CR 0/0/0/0 PR 14/14/14/16 SD 30/46/51/35	2.7/4.1/4.6/4.4	6.8/8.1/8.7/9.3	Neutropenia 18/12/16/22, anemia 4/0/4/8, thrombocytopenia 2/8/16/14, nausea + vomiting 2/2/0/10
Von Hoff *et al*[50]	Nab-PTX + GEM	I	67	CR 4 PR 42 SD 18	7.9	12.2	Neutropenia 67, thrombocytopenia 23, asthenia 21, neuropathy 15
Hosein *et al*[51]	Nab-PTX	II	19	CR 0 PR 5.3 SD 31.6	1.6	7.3	Neutropenia 32, febrile neutropenia 11, anemia 11
Kim *et al*[52]	PTX + 5-FU	II	28	CR 0 PR 10 SD 20	2.5[1]	7.6	Leukopenia 6, diarrhea 2, neuropathy 1

[1]mTTP: Median time to progression; CT: Chemotherapy; RR: Response rate; mPFS: Median progression free survival; mOS: Median overall survival; PTX: Paclitaxel; NR; Not reported; GPM: Paclitaxel loaded polymeric micelle, ET: Paclitaxel embedded in cationic liposomes; Nab-PTX: Paclitaxel protein-bound particles; 5-FU: 5-fluorouracil.

(Table 1). In a phase II trial conducted on 10 patients, no response was obtained, mPFS was 1.5 mo and mOS was 4.0 mo[35].

Combination chemotherapy with taxanes as salvage treatment gave disappointing results with a significant toxicity[37-39]. In these trials, docetaxel combined with capecitabine[37], with irinotecan[38], and with mitomycin plus irinotecan[39] resulted in a RR ranging between 0% to 9.7%, and mOS between 4.5-6.3 mo. The most common toxicity in these studies was grade 3-4 neutropenia observed in around 30%-32% of patients[37-39]. Two retrospective series reported the results of GTX regimen as salvage therapy in patients affected by pancreatic cancer[31,40]. The RR was 12%-15% and mOS was 5.7-6.7 mo[31,40]. Altogether, the response and survival figures observed with docetaxel-based combinations as salvage therapy in advanced pancreatic cancer were in the range reported with other regimens[41-45]. These drug combinations tested in the second line gave RR between 0%-24% and mOS between 3.7-6.2 mo[41,42,44,45]. The PEFG combination was the only regimen that reported a better mOS in second line therapy (8.3 mo) with an acceptable toxicity[43].

PACLITAXEL AND NEW PACLITAXEL FORMULATIONS

First-line therapy

Single agent: Single agent paclitaxel yielded a RR of 3% and a mOS of 5 mo in a series of 45 patients with advanced pancreatic cancer (Table 2)[10], while paclitaxel loaded with polymeric micelle obtained an overall RR of 6.7%, mPFS of 2.8 mo, and mOS of 6.5 mo in 56 patients with advanced pancreatic cancer[46].

Combination chemotherapy: EndoTAG™-1 (ET) is a cationic liposome membrane charging paclitaxel. This particular structure promotes the delivery of the drug in the tumor mass. Tumor endothelium lacks glycocalix which normally covers endothelial cells, so negative charges are exposed on the cell surface. Thus, the positive charges carried by liposomes is exposed and interact with the negative charges present on tumoral cells favouring the internalization of the drug into the tumor[47].

Löhr *et al*[48] tested ET in combination with gemcitabine *vs* gemcitabine alone in a four-arm randomized phase II trial on 212 patients affected by locally advanced or metastatic disease (Table 2). The treatment consisted of seven weekly infusions of standard gemcitabine alone or associated with twice-weekly ET at dosage of 11 (Endo11), 22 (Endo22) or 44 mg/mq (Endo44) for seven weeks. RR was comparable across the four treatment groups (14%-16%), the mPFS was longer in the gemcitabine plus ET arms (4.1, 4.6, and 4.4 for Endo11, Endo22, and Endo44, respectively) compared to gemcitabine group (2.7 mo). Also the mOS appeared to be better in the combination arms (from 8.1 to 9.3 mo) compared to single agent (mOS 6.8 mo). The treatment with gemcitabine and ET was well tolerated with a dose-dependent increase in grade 3-4 thrombocytopenia (from 8% to 14%), neutropenia (from 12% to 22%), and anemia (from 4% to 8%). Grade 3-4 febrile neutropenia was observed in 6% of the patients. No treatment-related neuropathy was observed in this trial. This study suggested that this new formulation of paclitaxel warrants further investigation to define its role in the treatment of pancreatic cancer.

Another paclitaxel formulation known as ABI-007 was tested against pancreatic cancer. ABI-007 (also

known as *nab*-paclitaxel), is a cremophor-free, albumin-bound 130-nm particle form of paclitaxel that does not require the use of cremophor-EL, thus avoiding the severe toxicities associated with this vehicle[49]. The albumin in nab-paclitaxel binds to gp60 (albondin) receptors and to caveolae resulting in the formation of caveoli transporting the drug across the endothelial cells to the tumor interstitial space. In pancreatic tumor stroma, secreted protein acid and rich in cystein (SPARC) protein, which is also called osteonectin, is overexpressed. SPARC interacts with the albumin of nab-paclitaxel enhancing the concentration of this drug into the tumor, which causes "stromal collapse", a phenomenon of depletion and collapsing of stroma, bringing tumor cells closer to each other and to blood vessel. A phase IB-Ⅱ study of ABI-007 in combination with gemcitabine was performed in metastatic pancreatic cancer (Table 2)[50]. The maximum tolerated dose (MTD) was 125 mg/mq for ABI-007 in combination with standard gemcitabine[50]. The PFS for the whole population of patients enrolled into the trial was 6.9 mo and the mOS was 10.3 mo, while in the group of 44 patients treated with ABI-007 at MTD, the mPFS was 7.9 mo and the mOS was 12.2 mo. A phase Ⅲ clinical trial of gemcitabine and ABI-007 combination *vs* standard gemcitabine is currently ongoing in patients with metastatic pancreatic cancer (NCT00844649).

Salvage therapy

Single agent and combination chemotherapy: ABI-007 at 100 mg/mq weekly for three weeks, out of every 4, was also tested as second line chemotherapy in 19 patients with progressive pancreatic cancer after previous gemcitabine-based therapy (Table 2)[51]. One partial response (5.3%) and six stable disease (31.6%) were reported. The mPFS and mOS were 1.6 mo and 7.3 mo, respectively. Grade 3 or 4 neutropenia, neutropenic fever and anemia occurred in 32%, 11% and 11% of patients, respectively.

Paclitaxel in combination with 5-FU was administered as salvage therapy to 28 patients with advanced pancreatic cancer after gemcitabine failure (Table 2)[52]. The RR was 10%, the mTTP 2.5 mo, and the mOS 7.6 mo. This regimen was well tolerated with grade 3-4 neutropenia in 21.4% of the patients, anemia in 3.6%, grade 4 neuropathy in 3.6%, and grade 3 diarrhea in 7.2% of the patients.

TAXANES PLUS RADIOTHERAPY

Pancreatic cancer is characterized by a high rate of both local and systemic failure. Chemoradiation was tested in stage Ⅲ disease with different drugs yielding a mOS between 8 to 11 mo and 1-year survival rate between 25% to 40%[53-55].

Due to their radiation-sensitizing properties[56], taxanes were also tested in combination with radiotherapy in locally advanced[57,58] and in resectable[59] pancreatic cancer. In locally advanced disease, paclitaxel and radiotherapy obtained RR of 26%, mOS of 8-11.2 mo and 1-year OS of 30%-43%[57,58]. These results were in the range reported with other drugs tested in combination with radiotherapy.

Conversely, paclitaxel-based chemoradiation as neoadjuvant therapy in resectable patients yielded disappointing results[59]. In fact, 46% of the patients suffered grade 3 toxicity (hematological, gastrointestinal, asthenia, anorexia, allergic reaction) and the mOS (19 mo) was inferior than expected with 5-FU based chemoradiation (25 mo)[60].

A phase Ⅱ trial randomized 20 patients with resectable and unresectable disease to receive docetaxel plus either continuous 5-FU or weekly cisplatin concomitant to radiotherapy. The enrolment was prematurely concluded due to poor preliminary results[61].

CONCLUSION

Pancreatic cancer is characterized by a dismal prognosis and limited therapeutic progress has been achieved in the past 30 years. Due to its intrinsic or rapidly acquired chemoresistance, the therapeutic armamentarium against pancreatic cancer is limited and there is an urgent need to individuate new active agents or regimens. Single agent gemcitabine, despite poor activity and modest impact on disease outcome, is still considered the standard treatment both in early and advanced stages of the disease[62]. Most combination regimens using gemcitabine-based doublets and including both conventional and targeted agents failed to significantly improve OS over gemcitabine alone[6,7,33,63,64] or yielded a statistically significant but clinically negligible benefit[65]. Interestingly, two phase Ⅲ trials showed that drug combinations including more than two agents may improve OS when compared with gemcitabine alone[32,66] and two clinical practice surveys suggested that the 4-drug regimens may be superior to gemcitabine/platinating-agent doublets[41,67].

In particular, the PEFG regimen and the combination of 5-fluorouracil, leucovorin, irinotecan, and oxaliplatin (FOLFIRINOX) yielded 1-year OS of 38%-48%[32,66]. The results obtained with these regimens should be generalized with caution due to the lack of confirmatory trials and, in the case of FOLFIRINOX, to the highly selected patients population, which is evident on the basis of the better than expected standard arm outcome and because 4 years occurred to enrol 342 patients in 48 centers (< 2 pts/center per year)[66]. Moreover, while PEFG toxicity profile was favourable[32,68], and the regimen was in fact feasible also in the adjuvant setting[69,70], grade 3-4 toxicity observed with FOLFIRINOX was remarkable particularly in the case of extra-hematological toxicity that may be barely acceptable in the context of a palliative therapy. In fact, the main reason for ending treatment was death in 85 (50%) patients in the FOLFIRINOX arm *vs* 75 in the gemcitabine arm, while fatigue was reported in 24% of the patients, vomiting in 15%, diarrhea in 13%, and neuropathy in 9%[66]. Altogether, these results are encouraging and do suggest that a nihilistic attitude towards pancreatic cancer is no

longer justified and that more aggressive treatment approach may partially overcome chemoresistance. As previously observed with other drugs, like gemcitabine, 5-FU, capecitabine, pemetrexed[4,6,71], the use of taxanes as single agent treatment, both upfront and as salvage therapy, showed moderate activity but did not obtain exciting results[8,9,15,35,36]. Not surprisingly and similarly to fluoropyrimidines and platinating agents, the inclusion of old generation taxanes in doublets with gemcitabine or cisplatin did not appear to produce better results than gemcitabine alone[19,20,23-27,29,37-39]. On the other hand, the inclusion of taxanes in combination with more than 2 drugs[22,30,31,40] seem to be more promising. Worth of note, unexpected radiological and biochemical response was observed in an exploratory subset analysis in patients with stage Ⅲ disease (60% radiological response in PDXG group *vs* 37% in PEXG group and major plus minor biochemical response of 80% *vs* 64%, respectively[22]). Furthermore, more patients in PDXG arm underwent to surgery with radical intent compared to PEXG arm (17% *vs* 6%) and neither resection margin nor nodal involvement was observed in the group treated with docetaxel. Furthermore, the new generation of taxanes, due to their unique chemical structure, are able to penetrate in tumor cell mass in high amount and apparently yields better activity than older taxanes. Accordingly, taxanes, and above all, new generation taxanes, appear to be suitable candidates for further testing to assess their role against pancreatic cancer in various clinical settings.

FUTURE PERSPECTIVES

Apart from the combination of ABI-007 with gemcitabine as first-line therapy in metastatic disease, which is currently being tested in a phase Ⅲ trial (NCT00844649), the role of multiple (i.e., more than two drugs) agents regimens should be addressed. In fact, the hypothesis of stromal depletion induced by ABI-007, if confirmed, may provide a robust rationale for combination polychemotherapy, due to better drug penetration into tumor. Furthermore, a larger effect may be expected in primary tumor where the stroma is more abundant[72-74]. A phase Ⅱ clinical trial is evaluating a combination of ABI-007 with gemcitabine, and GDC-0449, a hedgehog inhibitor, in patients with untreated metastatic pancreatic cancer in order to evaluate the PFS and the safety of this combination (NCT01088815). The hedgehog signalling pathway is involved in embryonic development, but is also activated in pancreatic cancer[75]. In preclinical model the inhibition of this pathway enhanced drug delivery to tumor cells by disrupting the desmoplastic stroma and increasing tumor vascularity[76]. The combination of gemcitabine, ABI-007 and GDC-009 could enhance the stroma collapse and increase the intratumoral concentration of chemotherapeutic drugs. Accordingly, neoadjuvant therapy in patients with stage Ⅲ disease, borderline resectable disease and resectable disease represents a potential field of investigation. Finally, ABI-007 may improve primary tumor oxygenation by inhibiting the formation of novel microvessel and by disrupting established microvessels thus increasing the therapeutic window of concomitant radiation therapy and targeted agents[77,78]. So, the next logical step is to evaluate a combination of anti-angiogenic therapy with ABI-007 in metastatic setting. Furthermore, the identification of new prognostic markers like SPARC could help both in understanding the molecular changes responsible for development and progression of pancreatic cancer and in identifying a subset of patients in which taxane-based therapy may have a more relevant impact on the outcome.

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FIELD OF VISION

Confounding factors affect the pathophysiology of eosinophilic esophagitis

Yoram Elitsur

Yoram Elitsur, Department of Pediatrics, Gastroenterology Division, Joan C. Edwards School of Medicine, Marshall University, Huntington, WV 25701, United States
Author contributions: Elitsur Y solely contributed to this manuscript.
Correspondence to: Yoram Elitsur, MD, Professor in Pediatrics, Department of Pediatrics, Gastroenterology Division, Joan C. Edwards School of Medicine, Marshall University, 1600 Medical Center Drive, Huntington, WV 25701, United States. elitsur@marshall.edu
Telephone: +1-304-6911300 Fax: +1-304-6911375
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Abstract

Eosinophilic esophagitis is a newly diagnosed esophageal disease in adult and children. The clinical and pathological characteristics of this disease have been established and were recently summarized in the expert clinical guideline published in 2011. In spite of the wide knowledge accumulated on this disease, there are many areas where scientific data are missing, especially in regard to the disease's pathophysiology. Recent publications have suggested that other confounding factors modify the disease and may affect its clinical-phenotypic presentation. Those factors may include place of living, air pollution, race, genetic factors and other. In the present report we discussed and review those confounding factors, the new developments, and what direction we should go to further advance our knowledge of this disease.

Key words: Eosinophilic esophagitis; Confounding factors; Race; Gender; Environment

Peer reviewers: Ferruccio Bonino, MD, PhD, Professor of Gastroenterology, Director of General Medicine 2 Unit, Director of Liver and Digestive Disease Division, Department of Internal Medicine, University of Pisa, University Hospital of Pisa, Via Roma 67, 56124 Pisa, Italy; Andrzej S Tarnawski, MD, PhD, DSc (Med), Professor of Medicine, Chief Gastroenterology, VA Long Beach Health Care System, University of California, Irvine, 5901 E. Seventh Str., Long Beach, CA 90822, United States

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INVITED COMMENTARY ON HOT ARTICLES

Eosinophilic esophagitis (EoE) is a chronic esophageal disease associated with allergy in adults and children. The clinical, endoscopic, and histological characteristics of EoE has been described and documented in several expert reports[1,2]. In spite of the knowledge accumulated on this newly discovered disease, double blind placebo control studies have demonstrated that only about 50% of the patients will respond to therapy (biologic, steroids)[3,4], demonstrating the existing gap between the clinical and/or basic knowledge, proposed therapy and the pathophysiology of EoE. The complete story of EoE in children is yet to be unfolded.

In a recent publication, Sperry *et al*[5] compared the clinical presentation of EoE in a cohort of adult and pediatric patients diagnosed with EoE. The authors suggested that African American (AA) patients were younger at diagnosis, presented with failure to thrive (FTT), and were less likely to have esophageal rings compared to the Caucasian (C) patients[5]. The paper has raised the possibility of other unknown confounding factors that may influence the clinical/phenotypic presentation of EoE in adults and children. Indeed, reviewing the

literature of EoE disease suggested that various factors may modify the disease incidence, clinical presentation, and/or clinical response to therapy. Those possible confounding factors are the topic of this paper (Table 1).

Epidemiological studies have showed that the disease's rate is unevenly distributed among adults and children living across the United States. The data showed that the prevalence of the disease in the North-Eastern states is higher in comparison with the South-Eastern states[6-8]. Moreover, further analysis demonstrated that EoE is more prevalent in urban/sub urban areas compared to rural areas[6,8,9]. The data suggested that there are environmental factors that modify EoE, but to date, those modifiers have not been clearly analyzed. The current geographic distribution may implicate air pollution as a modifier factor for EoE[10]. Indeed, animal and clinical studies have confirmed the essential role of aero-allergens in the pathophysiology of the disease[11-13]. Few investigators reported an increase incidence of EoE during the allergy seasons (low during winter, high during spring and fall)[14-16], but those findings were not confirmed by others. For example, in a recent preliminary report, Frederickson *et al*[17] reviewed esophageal biopsies in a cohort of 19 172 patients in Iowa City, IA, of whom 167 adults had EoE. The author reported a comparable monthly rate of EoE with no seasonal variation. Using a national pathology laboratory data base, the seasonal trend of 9995 adult patients was examined by others who reported a similar monthly and seasonal distribution of the patients diagnosed with EoE[18]. Similar results were described by investigators who performed an internet search to look for EoE and seasonal variation[19]. In a recent study, Hurrell *et al*[20] reported a higher prevalence of esophageal eosinophilia in adults who live within the colder geographic zones of the United States compared to those living in the warmer climates. They implicated that the specific flora grow in those areas generate highly potent aeroallergens, and is responsible for their findings. In contrast, the United States governmental environmental agency (Environmental Protection Agency, National Oceanic and Atmospheric Administration, National Park Service) reported the lowest air pollution in the northern geographic areas of the United States, those with colder climate rather than the warmer climate (http//airnow.gov).

In addition to the possible role of aeroallergens and pollution in the pathophysiology of EoE, clinical reports have documented the etiologic role of food allergy in this disease[3,11,21-23]. Indeed, the updated clinical guideline recommended checking food allergy in any patient who is newly diagnosed with EoE[1]. Moreover, in support of this role, eliminating the food allergens alone (i.e., hypoallergenic formula) resolved the clinical symptoms and reversed the histological changes typically seen in EoE[1,24-26]. In patients that would not accept this mode of therapy, topical or systemic steroids are suggested[1]. In a randomized, double-blind, placebo control study, EoE children were treated with Fluticasone propionate[3].

Table 1 Confounding factors and eosinophilic esophagitis

Factor	Ref.
Geographic distribution	[6-9,20]
Air pollution (aeroallergens)	[11-13]
Food allergy	[21-23]
Race (ethnicity)	[27-35]
Gender	[21,26]
Genetic factors	[22,23,39,40]

The authors showed that the children with no allergy had a better response to therapy compared to the children with allergy, pointing towards the role of food allergens as modifiers[3]. Indeed, the role of food allergens in the pathophysiology of EoE has been established, but we are still lacking the exact function of these modifiers. Are all food allergens equal? What about the EoE patients who tested negative for any allergy? Is their disease is more benign? Do they achieve symptom control easier? All these questions are still unanswered and need further investigation.

The different distribution of EoE among various ethnicities has been previously published in adults and children. For example, the clinical presentation of EoE in children reported from several United States centers showed a statistically higher disease rate in C compared to AA or hispanic[21,27-29]. Other reported similar findings in the adult population[5,30,31]. This discrepancy is further accentuated by the epidemiologic data showing that in spite of the wide global distribution of EoE, very few reports from the African continent or from the east Asian countries were reported[1,32-34]. In recent years we have assessed the different clinical presentation of EoE between AA and C children. In that study, we compared between AA children living in urban neighborhoods in New York City and C children who are living in rural West Virginia[35]. We reported that EoE in AA children presented at an earlier age, have more history of atopic features, and presented with significantly lower endoscopic features of EoE compared to the C children. Similarly, Sharma *et al*[36] reported that AA children are more likely to present with FTT, gastroesophageal reflux disease symptoms, and at a younger age compared to the C children at the same cohort. Sperry *et al*[5] compared the clinical presentation of EoE in a cohort of adult and pediatric patients diagnosed with EoE. The authors suggested that when compared with C children, AA children diagnosed at a younger age, present with FTT, and have less esophageal rings[5].

Overall, these data suggest that racial factor does play a role in EoE disease and may serve as a modifier in the disease phenotypic presentation. Further studies will be needed to fully establish the role of ethnicity in the pathophysiology of EoE.

Male sex is the predominant gender in EoE[1]. In large series, the male/female ratio was reported up to 3:1[21,37]. The effect of gender on EoE disease has not been well investigated and studies on this subject are

lacking. Rothenberg *et al*[38] suggested that the predominance of males in EoE may be related to the thymic stromal lymphopoietin receptor resided within the pseudoautosomal region 1 on the X and Y chromosomes (Xp22.3, Yp11.3). The author suggested that this region may explain the higher incident of male gender in EoE.

Similar to many other chronic diseases; i.e., inflammatory bowel disease, celiac disease, asthma, *etc*., genetic factors have a significant influence on the prognosis of EoE. Indeed, several investigators evaluated the genetic factors related to EoE. Few investigators reported that EoE tends to cluster in families[22,23], and other showed by genome-wide microarray expression analysis that EoE patients have a different spectrum of genes compared to children with gastroesophageal reflux disease or normal control[39]. Others showed that specific genes are closely associated with eosinophil's activation and are increased in patients with EoE[1,4,39,40]. In another study, Lu *et al*[41] identified 32 different miRNA which are specific for EoE, of which miRNA-21 and miRNA-223 were the most up-regulated, and decreased post steroid therapy. In a preliminary report, Gonsalves *et al*[42] reported that the esophageal epithelial barrier genes (Desmoglein 1) is lower in adults with EoE who did not respond to dietary therapy, suggesting a dysfunctional, leaking esophageal mucosa. The new data may explain the role of aeroallergens in the disease's pathophysiology, by permitting the absorption of allergens (aeroallergens or food allergens) through the tightly sealed epithelial layer of the esophagus, and/or may explain the quick absorption of topical steroid into the esophageal mucosa during therapy.

Overall, the genetic framework involved in the pathophysiology of EoE is emerging and the genes responsible for activation or suppression of the inflammatory process are slowly uncovered. Nevertheless, we still have a large gap of knowledge left to explain the different response to therapy in EoE patients who belong to a different gender, different ethnicity, or have different allergies.

In summary, eosinophilic esophagitis is a chronic disease of the esophagus with clinical and histological characteristics previously established and described[1]. As in many chronic diseases, the clinical and/or histological presentation may be altered by various environmental or genetic modifiers. Epidemiological and clinical reports have documented the different phenotypic presentation of EoE in different populations, but further investigation is needed to dissect those disease modifiers in order to be able to tailor therapy for the specific patient. In the era of personalized medicine these modifiers play a crucial role in the disease's prognosis, and future studies to address those factors are clearly warranted.

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FIELD OF VISION

Diagnostic criteria for autoimmune hepatitis in children: A challenge for pediatric hepatologists

Priscila Menezes Ferri, Alexandre Rodrigues Ferreira, Débora Marques Miranda, Ana Cristina Simões e Silva

Priscila Menezes Ferri, Alexandre Rodrigues Ferreira, Division of Pediatric Gastroenterology and Hepatology, Department of Pediatrics, School Medicine, Federal University of Minas Gerais, Minas Gerais 30130-100, Brazil
Débora Marques Miranda, Ana Cristina Simões e Silva, National Institute of Science and Technology of Molecular Medicine, Faculty of Medicine, Federal University of Minas Gerais, Minas Gerais 30130-100, Brazil
Author contributions: Ferri PM collected the materials and wrote the manuscript; Ferreira AR and Miranda DM discussed the topic and revised the text; Simões e Silva AC conceived the manuscript and made the general supervision.
Correspondence to: Ana Cristina Simões e Silva, MD, PhD, National Institute of Science and Technology of Molecular Medicine, Faculty of Medicine, Federal University of Minas Gerais, Avenida Alfredo Balena 190, Belo Horizonte, Minas Gerais 30130-100, Brazil. acssilva@hotmail.com
Telephone: +55-31-93600007 Fax: +55-31-34099772
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Abstract

Autoimmune hepatitis (AIH) is a progressive inflammatory liver disorder that is rare in children and adolescents. AIH has a broad clinical spectrum and a quick response to treatment with corticosteroids and immunosuppressive medication. The available diagnosis criteria have limitations and should be evaluated in pediatric populations. Recently, some studies reported that the 2008 simplified diagnostic criteria for AIH could be used in children with high sensibility and specificity. In addition, the authors reported that globulin and immunoglobulin G levels can be used interchangeably for diagnostic purposes. They also demonstrated that the 2008 simplified criteria fail in identifying patients with fulminant hepatic failure. Here, we discuss the limitations of the use of these criteria in pediatric patients and the requirement of more studies to improve the diagnosis of AIH in children.

Key words: Child; Autoimmune hepatitis; Liver diseases; Diagnosis; Autoimmunity

Peer reviewer: Ferruccio Bonino, MD, PhD, Professor of Gastroenterology, Director of General Medicine 2 Unit, Director of Liver and Digestive Disease Division, Department of Internal Medicine, University Hospital of Pisa, University of Pisa, Via Roma 67, 56124 Pisa, Italy

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INVITED COMMENTARY ON HOT ARTICLES

Autoimmune hepatitis (AIH) is particularly aggressive in children and progresses rapidly unless immunosuppressive treatment is promptly started. The recent publication by Mileti *et al*[1] on the validation of simplified diagnostic criteria for autoimmune hepatitis in children caught our attention for its updated theme, with great relevance to pediatrics and hepatologists. We highly recommend reading the manuscript.

AIH is a progressive inflammatory liver disorder that is rare in children and adolescents. It affects patients who have lost immune tolerance to liver self-antigens[2-4]. It predominates in females, and it is serologically characterized by high levels of transaminases and immunoglobulin G (IgG) and the presence of auto-antibodies and histologically characterized by interface hepatitis in the absence of a known etiology[3]. The age of onset of AIH ranges from 6 mo to 75 years; however, it is rare

before 2 years, and the incidence is higher between 10 and 30 years[3].

The clinical spectrum is broad; asymptomatic patients may only have laboratory abnormalities. Patients may also present with clinical symptoms similar to acute viral hepatitis and even severe liver failure (acute, chronic or fulminant)[3]. Auto-antibodies, such as antinuclear antibodies (ANA), anti-smooth muscle antibody (SMA) and anti-liver/kidney microsome type 1 (anti-LKM1), are important for the diagnosis of AIH. Based on the pattern detected, AIH can be classified into two types: type 1, in which ANA and/or anti-SMA are detected, and type 2, in which anti-LKM1 is detected[5].

Some histological findings can suggest the presence of AIH: piecemeal necrosis with periportal/periseptal lymphocytic infiltrate; interface hepatitis with the destruction of hepatocytes at the periphery of the lobule and erosion of the limiting plate; hepatic regeneration with "rosette" formation; and "bridging collapse", in which connective tissue collapses and expands from the portal area into the lobule[6].

Treatment with corticosteroids and immunosuppressive agents is usually efficient in controlling AIH[3]. Prednisone alone or combined with azathioprine is the main treatment, and it is aimed at reducing liver inflammation and inducing clinical remission and better survival rates. The treatment response is characterized by clinical improvement and the reduction of aminotransferase levels to normal or up two times the highest reference value[3].

Given the necessity to standardize the diagnosis and establish early treatment, in 1993, the International Autoimmune Hepatitis Group (IAIHG) created diagnostic criteria for AIH. Those criteria were revised in 1999 to improve specificity and simplify the scoring system, as cited in the discussion section of the manuscript[1,7]. Simplified diagnostic criteria were established in 2008 to make their use in clinical practice easier.

Mileti *et al*[1] selected 37 children under 21 years old with a diagnosis of AIH and 40 children diagnosed with other liver diseases and evaluated the sensitivity and specificity of the diagnostic criteria proposed in 2008. They found a sensitivity of 87% and a specificity of 89% for the 2008 criteria and also observed that these criteria did not rank patients with signs of fulminant hepatic failure (FHF) well. The authors also compared the use of IgG and serum globulin levels to grade the criteria and concluded that they may be used interchangeably without impairing the final score.

The use of 2008 diagnostic criteria might simplify the day-to-day work of hepatologists and might provide an interesting measure for pediatric patients. Based on the cited results and considering that autoimmune hepatitis is important in the differential diagnosis of liver disease in childhood, we discuss some important points that may affect the use of these criteria.

Use of diagnostic criteria in pediatric patients

In general, the adoption of diagnostic criteria is considered to be an attempt to standardize observations and clinical procedures for different centers with the main goal of a "common language". Diagnostic criteria should contain well-defined measures and should be easy to apply in clinical practice to facilitate disease classification, diagnosis and, consequently, treatment. Another important issue concerning diagnostic criteria is the possibility of distinguishing patients who need to be assigned to different therapies or management strategies, even if the diagnosis is not clearly established. This important feature of the diagnostic criteria will certainly allow early treatment, which might improve the outcome of patients.

For many diseases and clinical conditions, we already have diagnostic criteria that were usually established for the adult population and subsequently adapted to pediatric patients. The ideal situation would be that the diagnostic criteria used for children arise from studies on this age group.

Another important point is that diagnostic criteria raise the possibility of a disease, but in most cases, it is difficult to exclude other diagnoses. For example, the diagnosis of rheumatic fever with the criteria originally proposed by Jones[8] in 1944 includes a combination of arthritis, fever, and a high sedimentation rate in the presence of a recent Group A streptococcal infection. However, many children with juvenile arthritis also present with exactly the same features[9].

For AIH, the same problem occurs, with the criteria being initially established for adults and later adopted with changes for pediatric patients. In its first version in 1993, based on consensus from the IAIHG, the diagnosis of AIH in the pediatric population was not considered to require separate diagnostic criteria, as cited by Mileti *et al*[1]. Two other studies attempted to evaluate recent criteria (1999 and the simplified criteria created in 2008) in pediatric populations, and they had controversial results. Those studies are listed and compared with the Mileti *et al*[1] study in Table 1.

The authors generally concluded that the 1999 and 2008 diagnostic criteria could be used in children with good sensitivity and specificity in most cases, but they also showed some limitations. First, in children with the final diagnosis of primary sclerosing cholangitis (PSC), the study of Ebbeson *et al*[10] found that the 1999 criteria adequately scored these patients as "not AIH", while Hiejima *et al*[11] showed that using both criteria, all 5 children with PSC were graded as having AIH. Considering these findings and the possibility of AIH/PSC overlap syndrome in children, the use of gamma-glutamyl transferase could improve the specificity of the 1999 criteria but would not improve the 2008 criteria. Therefore, the recent recommendation of performing cholangiographic evaluation in all children with an initial diagnosis of AIH was added[12,13].

The second issue is the limitation cited by Mileti *et al*[1] regarding the reliability of the 2008 criteria in identifying patients with FHF. The acuteness and severity of FHF

Table 1 Summary of pediatric studies on the validation of diagnostic criteria for autoimmune hepatitis

Authors	Place and date	*n*	Diagnostics	Tested criteria	Results	Conclusions
Mileti *et al*[1]	United States, 2012	68	37 AIH for 1999 criteria/ 31 AIH for 2008 criteria 40 non-AIH	1999 and 2008	1999 criteria: 29 of 31 subjects (94%) as definite AIH; 2 of 31 subjects (6%) as probable AIH Simplified criteria: 25 of 31 subjects (81%) as definite AIH; 2 of 31 subjects (6%) as probable AIH The 2008 diagnostic criteria had a sensitivity of 87% and a specificity of 89% and did not identify 4 patients with AIH and fulminant hepatic failure	The 2008 criteria showed high levels of sensibility and specificity Patients with fulminant hepatic failure need the 1999 criteria Globulin and immunoglobulin G can be used interchangeably
Ebbeson *et al*[10]	Canada, 2004	28	21 AIH 4 PSC 3 ASC	1999	18 of 21 (86%) with AIH scored as definite AIH and 3 of 21 (14%) scored as probable All patients with isolated PSC scored as not AIH	The IAIHG scoring system has a use in children Using the GGT ratio may improve the specificity for children
Hiejima *et al*[11]	Japan, 2011	56	20 AIH 36 non-AIH liver diseases	1999 and 2008	Sensitivity and specificity of the 1999 criteria were 100% and 81%, respectively Sensitivity and specificity of the simplified criteria were 55% and 86%, respectively All 5 children with PSC were graded as having AIH by both criteria	The specificity of the simplified AIH criteria is high Simplified criteria could not differentiate between AIH and PSC and do not seem to be a reliable diagnostic tool in children

AIH: Autoimmune hepatitis; PSC: Primary sclerosing cholangitis; ASC: Autoimmune sclerosing cholangitis; IAIHG: International autoimmune hepatitis group; GGT: Gamma-glutamyl transferase.

demand a rapid and precise diagnostic definition. If the diagnostic criteria provide information in this special situation, treatment with corticosteroids could be started promptly. Thus, further research is important to identify factors or measurements that could improve the diagnosis of FHF.

The third issue is that the auto-antibodies used for diagnosis often have lower titers in children than the cut-off values considered positive in adults and utilized in both diagnostic criteria (1999 and 2008)[4]. The reactivity of ANA, SMA and anti-LKM1 is low; therefore, titers of 1/20 for ANA and SMA and 1/10 for anti-LKM1 can be considered relevant in children[4]. The 2008 and 1999 criteria consider titers above 1/40 to be significant, and the laboratory test is performed with a minimum dilution of 1/40; therefore, children with titers of 1/20 could be false negative for the tested auto-antibody. Therefore, in children with clinical history and a physical examination compatible with AIH, we should not exclude this diagnosis when negative auto-antibody results are obtained.

The fourth issue is the use of histological findings as one point of the diagnostic criteria for AIH. Histology was included in the 1993 and 1999 criteria, and each feature of the liver biopsy was scored as a separate item[7]. In contrast, the simplified criteria utilize only two parameters: histology compatible with AIH and histology typical of AIH[14]. The concern is that a biopsy is not possible at the beginning of follow-up in many pediatric cases because these patients usually exhibit significant liver dysfunction and coagulopathy. Thus, sometimes, clinicians do not have this information and face the necessity of initiating corticosteroid therapy without knowledge of the histological condition. Björnsson *et al*[15] questioned the importance of histology in the diagnosis of typical cases of AIH and concluded that the majority of patients with typical laboratory features of AIH are likely to have compatible liver histology. Not surprisingly, liver biopsy may reveal another hepatic disease that might affect clinical management. We agree that whenever possible, liver biopsy should be performed prior to initiating immunosuppressive therapy in patients with AIH. However, we also believe that if it is not possible to obtain initial histological findings, the initiation of treatment for highly suspected patients should not be delayed. Indeed, for the majority of cases with suggestive clinical features and compatible laboratorial data, the diagnosis of AIH can be reliably established in the absence of liver histology. Ultimately, liver biopsy should be performed, whenever possible, to differentiate other hepatic diseases and according to the guidelines of the American Association for the Study of Liver Disease Practice Guidelines for Autoimmune Hepatitis[13].

The last point to be addressed is the sample size of the Mileti *et al*[1] cross-sectional study, which was composed of just a few individuals classified with both criteria. Similar to other pediatric studies on AIH, the sample size is small. Additional studies should be performed with other populations to establish the best diagnostic criteria for pediatric AIH and to address all other points that require further elucidation in AIH and hepatic-related disorders.

In conclusion, the use of 2008 diagnostic criteria is an important tool in the diagnosis of AIH in children, and the study of Mileti *et al*[1] clearly corroborates this view. However, considering the existing criteria, more studies with larger series seem to be necessary to validate their use in pediatric patients. Finally, we believe that additional pediatric studies on AIH might allow for the clear differentiation between AIH and PSC, include

alternative ways to define fulminant hepatic failure and establish lower auto-antibody titers for pediatric patients.

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FIELD OF VISION

Asymptomatic pancreatic lesions: New insights and clinical implications

Martin Loos, Christoph W Michalski, Jörg Kleeff

Martin Loos, Christoph W Michalski, Jörg Kleeff, Department of Surgery, Klinikum rechts der Isar, Technische Universität München, 81675 München, Germany
Author contributions: All authors collected the material; Loos M drafted the article; Michalski CW and Kleeff J critically revised the article for important intellectual content; and all authors approved the final version of the manuscript.
Correspondence to: Jörg Kleeff, MD, AGAF, FACS, Department of Surgery, Klinikum rechts der Isar, Technische Universität München, Ismaninger Strasse 22, 81675 München, Germany. kleeff@tum.de
Telephone: +49-89-41405098 Fax: +49-89-41404870
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Abstract

Despite great efforts in experimental and clinical research, the prognosis of pancreatic cancer (PC) has not changed significantly for decades. Detection of pre-invasive lesions or early-stage PC with small resectable cancers in asymptomatic individuals remains one of the most promising approaches to substantially improve the overall outcome of PC. Therefore, screening programs have been proposed to identify curable lesions especially in individuals with a familial or genetic predisposition for PC. In this regard, Canto *et al* recently contributed an important article comparing computed tomography, magnetic resonance imaging, and endoscopic ultrasound for the screening of 216 asymptomatic high-risk individuals (HRI). Pancreatic lesions were detected in 92 of 216 asymptomatic HRI (42.6%). The high diagnostic yield in this study raises several questions that need to be answered of which two will be discussed in detail in this commentary: First: which imaging test should be performed? Second and most importantly: what are we doing with incidentally detected pancreatic lesions? Which ones can be observed and which ones need to be resected?

Key words: Pancreatic cancer; Early-stage pancreatic cancer; Asymptomatic high-risk individuals; Pre-invasive pancreatic lesions; Cystic pancreatic tumors; Screening; Computed tomography; Magnetic resonance imaging; Endoscopic ultrasound

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INVITED COMMENTARY ON HOT ARTICLES

With great interest we noticed the article published by Canto *et al*[1], which investigated the prevalence and characteristics of pancreatic lesions in high-risk individuals (HRI) for pancreatic cancer (PC).

Up to 10% of PC cases are attributed to a familial or inherited predisposition that can substantially increase the risk for PC[2-7]. For example, patients suffering from Peutz-Jeghers syndrome have a 132-fold increased risk for PC[8]. Other genetic predispositions include hereditary pancreatitis (*PRSS1* gene mutations, lifetime risk for PC of up to 40%)[9,10], the familial atypical multiple

mole melanoma syndrome (*p16/CDKN2A* gene mutations, 13-fold to 22-fold increased risk)[11,12], the Lynch syndrome (mismatch repair gene mutations, 8.6-fold increased risk)[13,14], familial adenomatous polyposis (*APC* gene mutations, 4.5-fold increased risk)[15,16], the familial breast-ovarian cancer syndrome (*BRCA1/2* gene mutations, 2.3-fold to 10-fold increased risk)[6,7,17,18], and individuals with a strong history of PC (at least two first-degree relatives with PC, 6.4-fold to 32-fold increased risk)[2,19]. For these, HRI screening programs have been proposed to detect early-stage pancreatic cancers or even pre-invasive lesions, which are potentially curable because once PC progresses into advanced stages the chance for cure decreases abruptly. In the study by Canto *et al*[1], pancreatic lesions were detected in 92 of 216 HRI (42.6%). The confirmed or suspected final diagnosis included branch-duct intraductal papillary mucinous neoplasm (IPMN) (n = 82), combined-duct IPMN (n = 2), main-duct IPMN (n = 4), and pancreatic endocrine tumor (n = 3)[1]. Such a high prevalence of pancreatic lesions is rare but has been reported before by Verna *et al*[20] who detected pancreatic lesions in 11 of 33 HRI (33.3%) and 14 of 31 HRI (45%) by magnetic resonance imaging (MRI) and endoscopic ultrasound (EUS), respectively. A possible explanation for the high diagnostic yield is the high quality of the screening tests and the highly experienced team of radiologists and gastroenterologists involved in the diagnostic work-up in both studies. On the other hand, it is well known that the prevalence of pancreatic lesions increases with age. Therefore, the age at which the screening test was performed is crucial. In the study by Canto *et al*[1], the mean age of HRI at screening was 56.1 years which is comparable to other published studies. When compared to individuals in the general population, the baseline diagnostic yield was significantly higher in HRI. Incidental cystic pancreatic lesions can be found in up to 2.8% in the general population[21] and this number will increase with better imaging tests. These findings point towards the importance of screening initiatives for HRI with high-resolution imaging tests but also illustrate the dilemma we are currently facing. The more diagnostic imaging studies we perform, the more lesions we will find - even in asymptomatic individuals without increased PC risk. The question that remains is what to do with these incidental lesions. How accurate is our interpretation of an incidental pancreatic lesion? Which lesions really represent a precursor of PC and will proceed to invasive cancer? How many of these lesions already carry incipient cancer? In 2006, the Sendai consensus proposed guidelines for the management of cystic lesions including IPMN and mucinous cystic neoplasms (MCN), which have been widely adopted[22]. However, the diagnosis and subsequent recommendation for pancreatic resection are based on imaging in combination with fine needle aspiration from cystic lesions and confirmation of the presumed diagnosis can only be made after surgical resection. A recent study published by Correa-Gallego *et al*[23] reported a series of 330 patients with incidentally discovered cystic neoplasms of the pancreas from a high volume center for diseases of the pancreas. One-hundred-thirty-six patients (41%) were operated on at diagnosis. Preoperative and final histological diagnoses were correlated[23]. The accuracy of preoperative diagnoses was only 64% for presumed branch-duct IPMN (32 of 50 cases)[23]. Ten of the 18 patients (20%) had an extension to the main duct leading to the final diagnosis of combined-duct IPMN[23]. The diagnostic accuracy for presumed MCN was also only 60% (18 of 30 cases)[23]. Of all patients who were operated on, 6 had an invasive carcinoma (2 branch-duct IPMN, 3 main-duct IPMN, and 1 MCN) and 19 patients had a carcinoma in situ (8 main-duct IPMN, 8 cystic pancreatic endocrine neoplasms, and 3 others)[23]. Therefore, correct interpretation of pancreatic lesions is still problematic and even with established guidelines choosing the adequate treatment remains challenging because the final diagnosis can only be verified after surgical resection.

Another problem is that IPMN are usually multifocal which has been addressed by the concept of the field defect of pancreatic duct instability[24-27]. Pancreata which harbour an IPMN are at increased risk of developing carcinoma. Several studies of patients with IPMN reported synchronous or metachronous invasive PC and these cancers were also present in areas distant from the index IPMN[28-30]. The most recent study by LaFemina *et al*[31] analyzed the prevalence and site of PC progression in 157 patients with suspected or confirmed IPMN who were initially selected for radiographic surveillance. After a median length of surveillance of 15 mo (range: 6-193 mo), 97 patients (62%) eventually underwent resection[31]. Surgical pathology confirmed 18 cases of invasive carcinoma (11%), which were diagnosed at a median of 24 mo after the initial diagnosis of IPMN[31]. Ten patients had main-duct IPMN (56%), 5 had branch-duct (28%), and 3 had combined-duct (17%) IPMN[31]. Four patients (22%) developed PC in a region of the pancreas distinct from the radiographically identified IPMN[31]. Miller *et al*[32] followed 153 patients after pancreatic resection for IPMN with clear resection margins. The authors found that 31 patients developed de novo IPMN in the pancreatic remnant and in 3 cases an invasive carcinoma was diagnosed[32]. Therefore, the confirmation of IPMN requires continuous surveillance of the entire pancreatic gland or of the pancreatic remnant after previous resection[32].

In this regard, Canto *et al*[1] attempted to answer the question of which imaging test should be performed for screening HRI and compared computed tomography (CT), MRI and EUS for detecting pancreatic lesions. The authors found that EUS and MRI detected pancreatic lesions better than CT[1]. The baseline diagnostic yield for EUS, MRI, and CT was 42.6%, 33.3% and 11%, respectively[1]. The authors' conclusion that EUS and MRI are currently the best initial tests for detecting early pancreatic lesions is supported by other studies[33-37]. Main limitations of CT include not only its poor sensitivity for small pancreatic lesions (< 10 mm) which

is important for screening for early pancreatic neoplasms but also the use of ionizing radiation which has recently raised concerns regarding the increased risk of radiation-related cancers associated with CT[38]. However, multi-detector CT remains the most widely used imaging modality because of its high accuracy for detecting solid tumors and staging of pancreatic malignancies, its cost effectiveness and its non-invasive nature. Furthermore, EUS and MRI are more cost-intensive and both tests are more dependent on the experience of the performing and diagnosing gastroenterologist and radiologist. Although the invasive nature of EUS as an endoscopic procedure further limits its role in a screening program, the possibility of EUS-guided fine-needle aspiration of pancreatic cystic lesions may be of diagnostic value especially when malignant cells can be detected. Future work with molecular analysis of cyst fluid, direct cystoscopy, and confocal laser endomicroscopy may further enhance its diagnostic accuracy[39].

Based on the high diagnostic yield of modern high-resolution imaging tests, it appears to be reasonable to routinely screen HRI. Based on current evidence, MRI (and EUS) should be the initial imaging tests to be performed. The question at what age screening of HRI should start has yet to be answered.

Although we have learned a lot in the past two decades about the nature especially of pancreatic cystic lesions, we are still facing a great challenge of how to manage incidental pancreatic lesions. Canto *et al*[1] suggest that the goal of PC screening and surveillance programs should be to detect and selectively treat asymptomatic high-grade precursor neoplasms rather than focussing on detection of invasive cancers. However, especially because IPMN constitute a heterogeneous group of pancreatic cystic neoplasms, a better understanding of the natural history of IPMN and its subtypes is necessary to distinguish lesions that need immediate surgical resection and those that can be safely observed. Not only a better understanding of patient characteristics and further progress in imaging tests are needed but also the identification of reliable biomarkers that can be used to identify pancreatic lesions that are about to proceed to PC in asymptomatic individuals.

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FIELD OF VISION

Opposite fates of fructose in the development of metabolic syndrome

Marta Alegret, Juan C Laguna

Marta Alegret, Juan C Laguna, Department of Pharmacology and Therapeutic Chemistry, School of Pharmacy, University of Barcelona, Institute of Biomedicine, 08028 Barcelona, Spain
Marta Alegret, Juan C Laguna, Biomedical Network Research Centre, Physiopathology of Obesity and Nutrition (CIBERobn), 08028 Barcelona, Spain
Author contributions: Alegret M and Laguna JC contributed equally to the writing of this manuscript.
Correspondence to: Dr. Juan C Laguna, Department of Pharmacology and Therapeutic Chemistry, School of Pharmacy, University of Barcelona, Institute of Biomedicine, Avda. Diagonal 643, 08028 Barcelona, Spain. jclagunae@ub.edu
Telephone: +34-93-4024530 Fax: +34-93-4035982
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Abstract

This short review comments on the recently published work of Ishimoto *et al* regarding the opposing effects of fructokinase C and A isoforms on fructose-induced metabolic syndrome in mice. The framework for the commentary is the preexisting background of epidemiological and experimental data regarding the association between ingestion of fructose, as present in sweetened beverages, and the development of metabolic syndrome. The work of Ishimoto *et al* clearly confirms the negative effect of fructose on lipid and glucose metabolism, independently from the amount of energy provided by the ingested sugar. It also confirms the absolute requirement of liver fructose metabolism, driven by fructokinase activity, in order to develop the full spectrum of metabolic syndrome alterations.

Key words: Lipid metabolism; Fatty liver; Obesity; Hyperglycemia; Dyslipidemia

Peer reviewers: Astrid van der Velde, PhD, AMC Liver Center, Meibergdreef 69-71, 1105 BK Amsterdam, The Netherlands; Andrzej S Tarnawski, MD, PhD, DSc (Med), Professor of Medicine, Chief Gastroenterology, VA Long Beach Health Care System, University of California, Irvine, CA, 5901 E. Seventh Str., Long Beach, CA 90822, United States; Ferruccio Bonino, MD, PhD, Professor of Gastroenterology, Director of General Medicine 2 Unit, Director of Liver and Digestive Disease Division, Department of Internal Medicine, University Hospital of Pisa, University of Pisa, Via Roma 67, 56124 Pisa, Italy

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INVITED COMMENTARY ON HOT ARTICLES

Non-communicable diseases continue to place an enormous burden on humanity, and have recently surpassed infectious diseases as the primary concern for human health. Among the former, cardiovascular events derived from the development of atherosclerosis associated with chronic metabolic diseases (such as obesity, type 2 diabetes mellitus and metabolic syndrome) are increasing all over the world. Besides genetic predisposition, two lifestyles options that are deeply rooted in affluent Western societies are fuelling this vicious trend: a lack of physical activity and the consumption of hypercaloric diets which tip people's energy balance to an overwhelming excess of ingested, unused energy. Dietary changes favor the consumption of processed, palatable, high-calorie-density foodstuffs over traditional ones. Among the former are the whole spectrum of sweetened beverages (fruit juices, sodas, milk shakes, *etc.*) which are sweetened with High Fructose Corn Syrup or sucrose. In both cases, such beverages offer the ingestion of large amounts of two simple sugars, glucose and fructose, in similar proportions[1,2].

When ingesting calories in liquid form, as in the case of the consumption of sugar-sweetened beverages, there is a lack of an appropriate compensatory response and thus no corresponding adequate reduction in the ingestion of calories provided by solid food. Therefore, it favors an excessive daily intake of calories. However, besides the increase in ingested calories, the particularities of fructose metabolism also need to be considered. Fructose is the carbohydrate with the greatest ability to induce hypertriglyceridemia, in part due to a more marked increase in hepatic lipogenesis than that resulting from a similar intake of glucose[3,4].

After entering liver cells, fructose is rapidly metabolized by the enzyme fructokinase to fructose-1-phosphate. Fructose feeding induces its own metabolism by increasing fructokinase expression. The product of fructokinase (fructose 1-phosphate) is further metabolized by the enzyme aldolase to the triose phosphates glyceraldehyde 3-phosphate and dihydroxyacetone phosphate. The former enters directly into the glycolytic pathway producing pyruvate, which is converted to acetyl-CoA and citrate inside the mitochondria, providing the carbon moiety for de novo synthesis of long-chain fatty acids. Meanwhile, dihydroxyacetone phosphate is converted into glycerol 3-phosphate, which provides the glycerol backbone of triglycerides. Since hepatic fructose metabolism bypasses the main rate-controlling enzyme of glycolysis, phosphofructokinase, a high flux of this carbohydrate to the liver results in a marked increase in lipogenesis and in the production of very low density lipoproteins. In contrast, glucose is first phosphorylated in position 6 by glucokinase, followed by isomerization to fructose-6-phosphate, which is converted by phosphofructokinase into fructose 1, 6-diphosphate before entering the glycolytic pathway. The progression of glucose to complete glycolysis is under the control of phosphofructokinase activity, which is very tightly regulated by the products of the glycolytic pathway, citrate and ATP. Thus, when large amounts of glucose are consumed, there is a feedback inhibition of glycolysis and glucose uptake, limiting pyruvate production, and the lipogenic effect is not as intense as in the case of fructose[5]. Furthermore, research from our laboratory has shown that fructose, but not glucose at a similar energy intake, can down-regulate the liver peroxisome proliferator activated receptor (PPAR) system, impairing fatty acid oxidation, and thus helping the accumulation of fatty acids for triglyceride accretion[6]. At least in male rats, the effect of fructose on the PPARα system is related to the induction of a clear state of liver leptin-resistance[7].

Fructokinase, and its capacity to respond to fructose increasing its expression[8-10] thereby facilitating the incorporation of fructose into liver metabolism, is of paramount importance in the manifestation of metabolic disturbances induced by fructose ingestion. For example, Ouyang *et al*[11] have shown that patients with non alcoholic fatty liver disease consume nearly approximately 2-fold to 3-fold more fructose than controls, primarily in the form of sweetened beverages, and present higher liver expression of fructokinase. In an experimental model of male rats supplemented with 10 % w/v fructose solution, we found that atorvastatin, a hypocholesterolemic drug, counteracts fatty liver and inflammation by reducing the fructose-related liver fructokinase induction[12].

All this background information is capital to clearly appreciate the real importance of the information provided by Ishimoto *et al*[13] in their paper published recently in Proceedings of the National Academy of Sciences. Fructokinase exists in two isoforms, fructokinase A and fructokinase C, with differentiated patterns of tissue expression. Fructokinase C is primarily expressed in liver, kidney and intestines, while the A isoform is expressed ubiquitously[14]. Due to the lower K_m of fructokinase C, this isoform is held to be mainly responsible for fructose metabolism[15]. By cleverly using the knock-out mice that developed themselves for both isoforms (KO-A/C) or only for isoform A (KO-A) and two patterns of fructose supplementation (15% and 30% w/v solutions of fructose in drinking water for 25 wk), Ishimoto *et al*[13] clearly delineated the roles of fructokinase A and fructokinase C in the induction of the manifestations of metabolic syndrome by fructose.

While wild-type mice supplemented with 15% fructose presented almost the full panoply of metabolic syndrome manifestations (obesity, hyperglucemia, hyperinsulinemia, hyperleptinemia and fatty liver), KO-A/C mice supplemented with 30% fructose, despite ingesting equivalent amounts of fructose, presented no manifestations of metabolic syndrome at all. In contrast, KO-A mice supplemented with 30% fructose, although ingesting the same amount of fructose as their matching wild-type controls, presented a more severe manifestation of metabolic syndrome than their wild-type counterparts. Ishimoto *et al*[13] carefully confirmed, by using recombinant fructokinase A, that this isoform, although having a low affinity for fructose, is capable of metabolizing fructose at a range of physiological concentrations.

In our opinion, three clear facts can be derived from the results presented by Ishimoto *et al*[13]: (1) KO-A/C mice were protected from developing metabolic syndrome, despite having a similar fructose intake and energy balance as their wild-type counterparts, which did indeed, develop metabolic syndrome. This experiment clearly demonstrates that the induction of metabolic syndrome by fructose does not depend on the amount of energy ingested (similar in KO-AC and wild-type control mice), instead it depends on the metabolism of ingested fructose, which clearly depends on the presence of fructokinase activity in tissues; (2) The fructokinase A isoform plays a physiological role in the metabolic fate of fructose. Its absence increases fructose concentration in plasma, even in animals ingesting only tap water; and (3) Furthermore, the absence of fructokinase A activity, as in KO-A mice, which shifts the whole metabolism of fructose to the C isoform, worsens the metabolic alterations induced by fructose ingestion. As mentioned above, fruc-

tokinase C is mainly expressed in liver and kidney and, to a lesser extend, intestines. If we consider that, after fructose ingestion, the sugar is rapidly and almost completely extracted by the liver through the glucose transporter 2[16], these results clearly point to the liver as the main target organ of fructose, and the one whose metabolic alteration finally results in the manifestation of metabolic syndrome in the whole organism. It would be very interesting to generate KO-C mice and supplement them with fructose in order to definitely confirm this claim.

While recognizing the problems of directly transposing experimental results obtained from animal models to the everyday-life of human consumption of sweetened beverages, the work of Ishimoto *et al*[13] demonstrates that fructose especial metabolism clearly matters as a key factor in the development of pathologies associated with the metabolic syndrome.

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FIELD OF VISION

Entry of hepatitis C virus into the cell: A therapeutic target

José Antonio Del Campo, Ángela Rojas, Manuel Romero-Gómez

José Antonio Del Campo, Ángela Rojas, Manuel Romero-Gómez, Unit for Medical and Surgical Management of Digestive Diseases, Center for Biomedical Research in Digestive and Liver Diseases Network (CIBERehd), Valme University Hospital, University of Seville, E-41014 Sevilla, Spain
Author contributions: Del Campo JA, Rojas A and Romero-Gómez M designed the study, performed bibliographic search and wrote manuscript and revision.
Correspondence to: Manuel Romero-Gómez, Professor, Unit for Medical and Surgical Management of Digestive Diseases, Center for Biomedical Research in Digestive and Liver Diseases Network (CIBERehd), Valme University Hospital, University of Seville, Avda. Bellavista s/n, E-41014 Sevilla, Spain. mromerogomez@us.es
Telephone: +34-95-5015761 Fax: +34-95-5015899
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Abstract

Several receptors have been identified as implicated on viral entry into the hepatocyte; and, this interaction between the virus and potential receptors could modulate infection, spontaneous viral clearance, persistence of the infection and the widespread of the virus as outbreak. Nevertheless, the playing role of each of them remains controversial. The Niemann-Pick type C1 like 1 gene (*NPC1L1*) receptor has been recently implicated on hepatitis C virus (HCV) entry into the cell and ezetimibe, an anti-cholesterol drug seems to block that, emerging the idea to control hepatitis C outbreak modulating lipid-related receptors. Hepatitis C infection seems to modulate lipid metabolism according to host genetic background. Indeed, it circulates like a lipoviroparticle. The main aim of this field of vision would be to discuss the role of hepatocyte receptors implicated on virus entry, especially NPC1L1 and the therapeutic options derived from the better knowledge about HCV-lipids- receptors interaction.

Key words: Hepatitis C virus entry; Niemann-Pick type C1 like 1 gene; Lipid metabolism; Ezetimibe

Peer reviewer: Ferruccio Bonino, MD, PhD, Professor of Gastroenterology, Director of General Medicine 2 Unit, Di-rector of Liver and Digestive Disease Division, Department of Internal Medicine, University Hospital of Pisa, University of Pisa, Via Roma 67, 56124 Pisa, Italy

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INVITED COMMENTARY ON HOT ARTICLES

Viral entry is the first step of virus-host cell interactions and, is a highly orchestrated process involving several viral and host cell factors. Indeed, the virus is able to escape from neutralizing antibodies and promote direct cell-cell transmission. Understanding the mechanisms of viral entry and escape is a prerequisite to define the viral and cellular targets that will give broad protection against hepatitis C virus (HCV) infection. HCV is an enveloped single-strand RNA virus that mainly targets hepatocytes. Due to the difficulty to grow HCV *in vitro* and the species specificity of this virus, surrogate model systems have been developed to study HCV entry into hepatocytes: recombinant envelope glycoproteins[1], HCV-like particles[2], HCV pseudo-particles[3,4] and recombinant infectious HCV[5-7] have been used to study the interactions of the viral envelope with human hepatoma cells or primary human hepatocytes. Four cellular factors have been described as essential for HCV entry (Figure 1): the tetraspanin molecule CD81, the scavenger receptor class B member I and the tight junction proteins claudin-1 and occludin[8]. Interestingly, neutralizing antibodies against CD81 are able to block HCV entry *in vitro* and also in im-

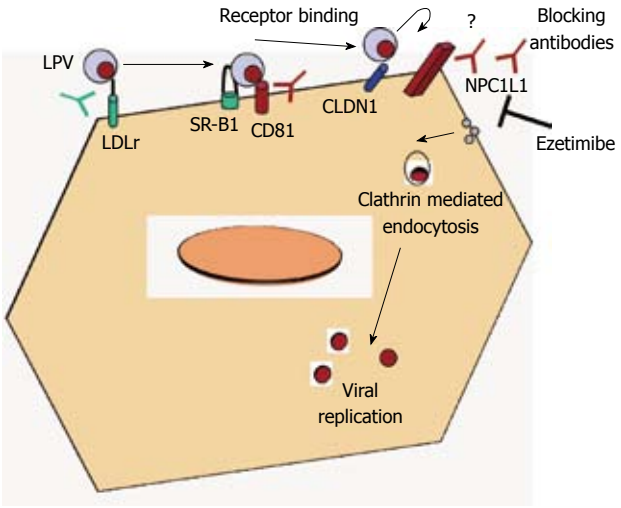

Figure 1 Schematic representation of viral receptors in hepatocytes. Possible mechanisms of viral entry blockade are also depicted by using specific antibodies or inhibitory drugs. LDLr: Low-density lipoprotein receptor; NPC1L1: Niemann-Pick C1-like 1 cholesterol absorption receptor; SR-B1: Scavenger receptor class B type 1; LPV: Lipoviroparticle; CLDN1: Claudin-1.

munodeficient mice transplanted with human hepatocytes, the best currently available small animal model of HCV infection[9]. Neutralizing antibodies against the HCV envelope proteins E1 and E2 could also represent a promising approach to avoid HCV infection, but the main challenge here is the enormous genetic variability of the virus. The requirement for sequential interactions between the viral envelope and key host receptors/co-receptors may provide new drug targets that could be exploited by small-molecule inhibitors. Recently, Syder *et al*[10] discovered and optimized a series of 1,3,5-triazine compounds that are potent, selective and non-cytotoxic inhibitors of HCV entry. Representative compounds fully suppress both cell-free virus and cell-to-cell spread of HCV *in vitro*. To date, only one oral HCV entry inhibitor with a defined mechanism of action, ITX-5061, has entered clinical testing (phase 2a)[11]. ITX-5061 binds directly to SR-B1 and blocks a key post-binding step in the viral entry process. In chronically infected patients we cannot find a single isolate of HCV but rather a population of related yet different viral variants (quasispecies), containing a vast repertoire of preformed variants that allow rapid escape from selective pressures such as neutralizing antibodies or anti-viral drugs. Recently, two well-known molecules have been shown to inhibit HCV entry: the green tea catechin epigallocatechin-3-gallate (EGCG) and the tyrosine kinase inhibitor erlotinib. Erlotinib blocks HCV entry by inhibition of the activity of the epidermal grow factor -receptor which is required for formation of CD81-claudin-1 co-receptor associations[12]. EGCG inhibits viral attachment to the target cell as well as cell-to-cell transmission between adjacent cells.

Role of Niemann-Pick C1-like 1 cholesterol absorption receptor in viral entry

Sainz *et al*[13] have recently discovered a novel surface receptor involved in HCV entry, the Niemann-Pick C1-like 1 cholesterol absorption receptor (NPC1L1), a 13transmembrane-domain cell surface cholesterol-sensing receptor, expressed on the apical surface of intestinal enterocytes and human hepatocytes, including Huh7 cells, is responsible for cellular cholesterol absorption and whole-body cholesterol homeostasis. NPC1L1 was first identified as a homolog of Niemann-Pick C1 protein[14], the deficiency of which causes Niemann-Pick disease type C1, a genetic disorder characterized by intracellular accumulation of unesterified cholesterol in the endosomal/lysosomal system of neurons that causes neurodegeneration and premature death[15,16]. Human *NPC1L1* gene maps to chromosome 7p13, spans 29 kb, encodes a 5 kb mRNA and predominantly produces a protein of 1332 amino acids[17]. NPC1L1 locates to the brush border membrane of the enterocyte and the canalicular membrane of the hepatocyte. Biliary cholesterol, which is secreted into bile by hepatocytes, accounts for more than two thirds of the total amount of cholesterol in the gut lumen. Since the cholesterol is water-insoluble, it is delivered to the brush border membranes by bile salt micelles.

These authors have shown that using NPC1L1-specific antibody, HCV infection was reduced in a similar way that CD-81 specific antibodies did. Ezetimibe is a 2 azetidinone-class drug that has been approved by the Food and Drug Administration as a cholesterol-lowering medication[18]. Sainz *et al*[13] also shown the role of NPC1L1 receptor on HCV infection *in vivo*, using immunodeficiency mice repopulated with human hepatocytes and treated them *via* oral gavage with ezetimibe. They have demonstrated that ezetimibe treatment delayed the establishment of HCV infection in mice pretreated for 2 wk, confirming the ability of this drug to inhibit HCV infection *in vivo*. However, ezetimibe concentration in those experiments was high (30 μmol/L). That means for a 70 kg weight adult to ingest 84 ezetimibe 10 mg tablets per day, when the usual doses for ezetimibe is one 10 mg tablet per day. So far, an ezetimibe based therapy for HCV does not seem suitable. Probably the use of antibodies against NPC1L1 would be a better alternative.

Lipid metabolism and HCV infection

HCV infection is tightly associated with alterations in lipid metabolism and lipids have been shown to play important roles during the viral replication cycle[19,20]. Indeed, recent studies based on transcriptome and proteomic analyses have demonstrated that expression of host genes involved in the biosynthesis, degradation and transport of intracellular lipids is profoundly altered upon infection. The expression of sterol regulatory element binding proteins, which control transcription of genes required for cholesterol biosynthesis, is stimulated by HCV infection. In agreement with this, the expression of fatty acid synthase (FASN) and other genes related to the synthesis and transport of fatty acids is upregulated in infected cells[21,22]. Moreover, the inhibition of FASN

activity blocks HCV RNA replication and production of infectious virus particles[23]. Finally, expression of genes regulating geranylgeranylation of cellular proteins important for HCV replication is also upregulated in HCV infected cells[21] .

HCV from infected patients (sera) can be precipitated with antibodies against lipoproteins (LP), indicating that HCV circulates associated to LP. Removal of LP by apheresis reduced HCV RNA level by 77%, suggesting that most of the viral particles are tightly associated with lipoproteins[24]. Two different studies[25] suggest that infectious HCV particles are highly associated with LP. Lipoproteins are easily endocytosed, supporting the hypothesis that HCV can use this association to LP to adhere the cell and subsequently enter into the host cell by endocytosis rather than direct fusion to the membrane[26].

Lipids droplets are necessary for the lipoviroparticle formation. HCV is hypothesized to initiate assembly in close association with lipid droplets by coating lipid droplets with the core protein and bringing together nonstructural (NS) and structural proteins in a NS2-dependent manner[27-29]. Following capsid assembly, nascent virions bud into the lumen of the endoplasmic reticulum (ER) where the glycoproteins E1/E2 reside in addition to the very low density lipoprotein (VLDL) secretion machinery. HCV is infectious upon envelopment at the ER, and it is thought that apolipoprotein E (ApoE) is acquired early during assembly because knockdown of ApoE reduces intracellular and extracellular virus; also NS5A interacts with ApoE[30,31]. Core proteins disturb microsomal triglyceride transfer protein (MTP) activity in the hepatocyte[32] and it has been described that NS5A could be interfered with MTP function. MTP is an essential chaperone for the assembly of VLDL, which transfers triglyceride, phospholipids, and cholesterol from the hepatocytes. Reduced activity of MTP results in decreased secretion of VLDL, leading to lipid accumulation. This fact could explain development of steatosis.

The low-density lipoprotein receptor (LDLr) was proposed as a potential entry factor for HCV[33], however, its implication in virus entry remains unclear. Moreover, by using HCV particles isolated from patients, a correlation has been shown between the accumulation of HCV RNA into primary hepatocytes, expression of LDLr messenger RNA, and LDL entry[34]. The potential involvement of the LDLr in HCV entry has also been reported in the HCVcc system[35]. Albecka *et al*[36] have shown that HCV particles can interact with the LDLr. However, this interaction does not necessarily lead to a productive infection. Furthermore, those data indicate a role for the LDLr as a lipid-providing receptor, which modulates viral RNA replication.

Quercetin, an abundant flavonoid found in fruits and vegetable, has been implicated in lowering the risk of cardiovascular disease that is often associated with high plasma levels of LDL cholesterol. Quercetin was found to inhibit NS3 activity in a specific dose-dependent manner in an *in vitro* catalysis assay, also inhibiting HCV RNA replication in the subgenomic HCV RNA replicon system and virus production in the HCV infectious cell culture system[37]. Gonzalez *et al*[38] have shown the marked reduction in viral production imparted by heat shock proteins synthesis inhibitor Quercetin. The low toxicity and pharmacokinetics of Quercetin are well known, and it has been approved for other uses in clinical trials. In fact, a phase I study evaluating the safety and tolerability of Quercetin in hepatitis C patients who have contraindications to standard antiviral treatment started last year (www.clinicaltrials.gov).

Administration of the exogenous interferons (IFNs) alpha, beta, and gamma in the setting of treatment for chronic HCV infection and other conditions has been shown to lower LDL cholesterol and raise triglyceride levels in VLDL, concomitant with suppression of lipoprotein lipase[39,40]. Li *et al*[41] have shown an association between rs12979860 genotype and host serum lipid levels, suggesting a relationship between endogenous IFN response and lipids. They hypothesize that the IFN-lambda rs12979860 CC responder genotype, which was associated with both increased likelihood of treatment response and higher LDL cholesterol levels in the studied cohort, is associated with lower IFN-lambda activity or lower intrahepatic IFN signaling gene expression. Our results indicate that LDL and total cholesterol levels were higher in patients infected with HCV genotype 1 harbouring the favourable genotype for interleukin 28B gene (Del Campo *et al*[29] unpublished data). These results suggest that observed associations are directly related to HCV-host interactions instead of a direct effect of this locus on lipid metabolism. At least in part, this host factor could select virus infection and promote chronic infection or spontaneous clearance according to viral genotype and lipid metabolism interplay.

Final remarks

HCV entry is a highly orchestrated process involving several viral and host cell factors, affecting infection, spontaneous viral clearance, persistent infection and widespread, and thereby offering multiple novel targets for antiviral therapy. A recently discovered novel surface receptor involved in HCV entry, NPC1L1, is responsible for cellular cholesterol absorption. This receptor arose as a new therapeutic target for HCV infection, since specific antibodies can block HCV entry. Ezetimibe treatment delayed the establishment of HCV infection in an animal model, emphasizing the relevance of the interaction between the host lipid metabolism and the establishment of a persistent infection, although utilized doses could not be translate to clinical practice. Host and virus genetic variation together with the interaction with hepatocyte receptors were assumed to explain the heterogeneity in HCV outcomes across individuals.

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TOPIC HIGHLIGHT

Toru Ishikawa, MD, *Series Editor*

Early administration of branched-chain amino acid granules

Toru Ishikawa

Toru Ishikawa, Department of Gastroenterology and Hepatology, Saiseikai Niigata Daini Hospital, Niigata 950-1104, Japan
Author contributions: Ishikawa T contributed solely to this review.
Correspondence to: Toru Ishikawa, MD, PhD, Department of Gastroenterology and Hepatology, Saiseikai Niigata Daini Hospital, 280-7 Teraji, Niigata 950-1104, Japan. toruishi@ngt.saiseikai.or.jp
Telephone: +81-25-2336161 Fax: +81-25-2338880
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Abstract

The effect of malnutrition on survival in patients with decompensated liver cirrhosis has not been well defined. Nutritional intervention with branched-chain amino acid (BCAA) can increase serum albumin concentration in patients with decompensated cirrhosis but its effects on survival are unclear. The BCAA to tyrosine ratio (BTR) is a surrogate marker (the normal range of BTR is between 4.41 and 10.05, and a Fischer's ratio of 1.8 corresponds to a BTR of 3.5) in patients with decompensated liver cirrhosis, and BCAA inhibits hepatic carcinogenesis in patients with compensated cirrhosis. This review discusses data regarding the effect of early administration of BCAA granules based on the ratio of BCAA to BTR on prognosis in patients with cirrhosis.

Key words: Branched-chain amino acid to tyrosine ratio; Branched-chain amino acid granules; Liver cirrhosis; Nutritional intervention; Malnutrition; Quality of life; Albumin; Cancer onset

Peer reviewers: Dr. Richard Parker, Department of Gastroenterology, Sandwell and West Birmingham NHS Trust, Dudley Road, Birmingham B1 2AF, United Kingdom; Ilker Tasci, Department of Internal Medicine, Gulhane School of Medicine, GATA Ic Hastaliklari Bilim Dali, 06018 Etlik, Ankara, Turkey

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INTRODUCTION

The liver plays a key role in nutrient metabolism, and patients with cirrhosis may develop various metabolism and nutrition disorders. In fact, many cirrhosis patients suffer from protein-energy malnutrition (PEM)[1], which is particularly pronounced in the decompensated stage. Correction of PEM can improve prognosis in patients with decompensated cirrhosis[2]. Patients with cirrhosis and decreased plasma branched-chain amino acid (BCAA) levels can develop PEM with increased catabolism[3].

PEM is associated with a high morbidity and mortality due to an increased risk of life-threatening complications, resulting in poor survival and poor quality of life (QoL)[4,5].

SIGNIFICANCE OF BCAA SUPPLEMENTATION

BCAAs are a group of essential amino acids, including valine, leucine, and isoleucine. A low plasma level ratio of BCAAs to aromatic amino acids suggests the presence of liver cirrhosis, and BCAA supplementation was originally developed in order to normalize the patient's amino acid profile and nutritional status[6].

This article summarizes the findings of previous studies to determine whether nutritional intervention with a granulated BCAA preparation can contribute to improved prognosis of patients with PEM and cirrhosis. In Japan, oral BCAA preparations are used in nutritional therapy to correct protein and amino acid abnormalities

in patients with cirrhosis. Moreover, enteral nutrition guidelines published by the European Society for Clinical Nutrition and Metabolism list BCAA supplementation as a grade B recommendation in the treatment of advanced cirrhosis[7].

BCAA supplementation effectively increases Fischer's ratio and improves hypoalbuminemia in patients with liver cirrhosis[8]. In addition, not only does BCAA serve as components of albumin, the activation by *L*-leucine of mammalian target of rapamycin in hepatocytes and the subsequent activation of albumin mRNA transcription and protein synthesis in ribosomes are thought to constitute the mechanism for albumin increase[9,10]. This increase in albumin has been verified in various clinical trials to be effective for improving hypoalbuminemia[8,11].

Oral BCAA preparations come in two dosage forms: enteral nutrition formulas (or elemental diet products) for liver failure and oral BCAA granules. In 1996, an oral BCAA granule product containing valine, leucine, and isoleucine (Val, Leu, Ile) in a composition ratio of 1:2:1.2 was marketed in Japan (Livact®, Ajinomoto Pharmaceuticals, Tokyo, Japan). This product is indicated for decompensated cirrhosis patients who have hypoalbuminemia despite adequate dietary intake.

IMPROVEMENT OF PROGNOSIS AND INHIBITION OF HEPATOCAR-CINOGENESIS

An Italian research group reported a multicenter randomized trial in which a total of 174 patients with advanced liver cirrhosis were given BCAA as a supplement for 1 year and its effects were compared with administration of lactalbumin or maltodextrin[12]. Long-term use of a BCAA granule preparation has been reported to increase serum albumin and to inhibit the incidence of events related to poor prognosis[8,12].

Moreover, Marchesini *et al*[12] analyzed whether oral BCAA might prevent progressive liver failure and improve nutritional parameters and quality of life.

They conclude that long-term nutritional supplementation with oral BCAA is useful to prevent progressive hepatic failure and to improve health status ,and recommend that new formulas are needed to increase compliance. An oral BCAA granule (Livact®, Ajinomoto Pharmaceuticals, Tokyo, Japan) is the form of small uniform granules, which reduces BCAA-induced stimulation of taste buds and contributes to improve compliance.

Kobayashi *et al*[13] conducted a study of patients with compensated cirrhosis caused by hepatitis C. After a mean follow-up of 3.2 years, the authors reported that the incidence of hepatocellular carcinoma (HCC) among men with a baseline serum albumin level of 3.6 to 4.0 g/dL tended to be lower in the BCAA granule treatment group than in the control group. A large-scale postmarketing clinical study conducted at 89 sites in Japan to determine the effects of BCAA granules on the prognosis of cirrhosis patients [Long-term Survival Study (LOTUS)] demonstrated that the onset of complications associated with poor prognosis (i.e., liver failure, ruptured esophageal varices, HCC, and death) was significantly lower among patients in the BCAA granule treatment group than in the dietary therapy group (hazard ratio: 0.67; 95% CI: 0.49-0.93)[8]. Furthermore, stratified analysis of the LOTUS study for groups at high risk for HCC, specifically, patients with a body mass index $\geqslant$ 25 kg/m^2 or elevated alpha-fetoprotein, showed that BCAA granules had an inhibitory effect on the incidence of HCC[14]. Meanwhile, a study by Tsuchiya *et al*[15] on radical therapy for HCC patients reported that long-term treatment with BCAA granules reduced the rate of the third and subsequent relapses of HCC and improved the cumulative rate of survival in patients with baseline serum albumin $\leqslant$ 3.5 g/dL. Sato *et al*[16] undertook a comparative study of dietary control protocols and found that a BCAA granule product and an enteral nutrient for liver failure (or an elemental diet product) had similar effects in terms of improving or maintaining serum albumin and preventing the onset of hepatic encephalopathy. The authors reported that, despite control of the total dietary energy intake, an increase in glycosylated hemoglobin and other markers of abnormal glucose tolerance occurred in the enteral nutrient group, whereas these unfavorable changes were not observed in the BCAA granule treatment group[16].

Moreover, Hayaishi *et al*[17] reported that Oral BCAA supplementation is associated with reduced incidence of HCC in patients with cirrhosis.

Based on these findings, a BCAA granule preparation (Livact®) has been now recommended in the guidelines for the treatment of liver cirrhosis by the Study Group for the Standardization of Treatment of Viral Hepatitis Including from the Ministry of Health, Labour and Welfare in Japan in order to increase serum albumin in cirrhosis patients with the aim of reducing the onset of cancer[18] (Table 1).

IMPORTANCE OF CONTINUED ADMINISTRATION

On the other hand, because some patients do not exhibit any increase in serum albumin after taking BCAA granules, further research has been conducted on the dietary intake and baseline characteristics of these patients. Yatsuhashi *et al*[19] reported that the anti-hypoalbuminemic effect of BCAA granules was not influenced by dietary intake and that continued use of BCAA significantly reduced the incidence of ascites and edema even in patients whose serum albumin did not respond to BCAA granule treatment.

One possible explanation for the significant decline in ascites and edema in the unchanged serum albumin group could be that the BCAA granules improved albumin quality. The sulfhydryl group of the cysteine 34 residue in human serum albumin can exist in a reduced state (reduced albumin) or oxidized state (oxidized albumin). In patients with chronic liver disease, however, the pro-

Table 1 Studies of outcome by branched-chain amino acid administration for cirrhotic patients

Authors	No. of cases	Study time	Outcome
Muto *et al*[8]	646	2 yr	Improving event-free survival, serum albumin concentration, and QoL
Kobayashi *et al*[13]	40	168 wk	Inhibiting hepatic carcinogenesis
Muto *et al*[14]	646	2 yr	Reducing the risk for liver cancer
Fukushima *et al*[21]	7	8 wk	Improving the oxidized/reduced state of serum albumin

QoL: Quality of life .

portion of oxidized albumin increases as the condition progresses[20]; this is associated with body fluid retention, such as ascites and edema. Furthermore, BCAA granules reduce the ratio of oxidized albumin in decompensated cirrhosis patients[21]. These findings suggest that the use of BCAA granules is important in maintaining serum albumin and that its continued use can improve the prognosis of patients with decompensated cirrhosis[22-25].

BCAA TO TYROSINE RATIO VALUE, AN IMPORTANT INDICATOR IN EARLY ADMINISTRATION OF BCAA GRANULES

However, the therapeutic effects of BCAA granules can take longer to appear in patients with advanced decompensated cirrhosis, making it important to determine the optimal timing for administration. After examining the biochemical test results of decompensated cirrhosis patients, Kato *et al*[26] identified the following four characteristics of uncompensated cirrhosis, and thus, the proper time to begin administration of BCAA granule preparations: (1) serum albumin $\leqslant$ 3.5 g/dL; (2) BCAA to tyrosine ratio (BTR) $\leqslant$ 3.5; (3) prothrombin activity $\leqslant$ 60%; and (4) platelet count of $\leqslant$ 100 000/mm^3. This in turn led to the search for markers of cirrhosis in order help facilitate determination of the optimal timing to initiate treatment with BCAA granules.

Cirrhosis patients exhibit shifts in their plasma free amino acid concentration, marked declines in BCAA (Val, Leu, Ile), and increases in aromatic amino acids (AAA; tyrosine, phenylalanine) and methionine, whereas their Fischer's ratio (ratio of molar concentrations of BCAA: AAA) or BTR declines in conjunction with disease severity. Fischer's ratio has long been used to analyze plasma free amino acids[27], but the BTR is a simpler method. Azuma *et al*[28] reported that the BTR based on an enzymatic method serves as an alternative to Fischer's ratio and is a potential indicator of liver disorders as well as subsequent chronic liver disease progression.

In the event of malnutrition, the BTR also declines before the serum albumin declines; therefore, determining the BTR is useful for the early detection of potential hypoalbuminemia. In other words, calculating the BTR enables the prediction of serum albumin level changes[29] and therefore allows determination of the appropriate time to administer BCAA granules. Given this time lag between decreases in serum albumin and BTR, monitoring of BTR needs to be done separately from that of albumin when considering prognostic factors for decompensated cirrhosis. The benefits of administering an oral BCAA preparation in patients with decreased BTR have already been reported in a large-scale clinical study[8]. This also implies that the BTR has considerable potential as a prognostic factor of HCC in decompensated cirrhosis patients. In fact, many reports performed the usefulness of BCAA for the treatment of hepatocellular carcinoma[30-39]. So, BTR may be useful as an indicator of prognosis in patients with HCC[40]. Early administration of BCAA granules based on the ratio of BCAA to tyrosine can improve the prognosis of decompensated cirrhosis.

In conclusion, BCAA supplementation for liver disorders may be expected not only to increase serum albumin, but also to exert other effects, such as prolongation of survival among liver cirrhosis patients, prevention of liver cancer, and enhancement of QoL. However, in cases of severe decompensated liver cirrhosis, determination of the timing of administration is also an important issue because BCAA granules take time to take effect. The decrease in BTR precedes reductions in serum albumin. While early therapeutic intervention with BCAA granules can help improve the prognosis of patients with decompensated cirrhosis and low BTR, more research and analysis are needed to fully explore the novel effects of BCAA granule preparations.

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GUIDELINES FOR CLINICAL PRACTICE

Donation after cardio-circulatory death liver transplantation

Hieu Le Dinh, Arnaud de Roover, Abdour Kaba, Séverine Lauwick, Jean Joris, Jean Delwaide, Pierre Honoré, Michel Meurisse, Olivier Detry

Hieu Le Dinh, Arnaud de Roover, Pierre Honoré, Michel Meurisse, Olivier Detry, Department of Abdominal Surgery and Transplantation, University Hospital of Liège, University of Liège, 4000 Liège, Belgium
Abdour Kaba, Séverine Lauwick, Jean Joris, Department of Anesthesia and Intensive Care Medicine, University Hospital of Liège, University of Liège, 4000 Liège, Belgium
Jean Delwaide, Department of Hepatology and Gastroenterology, University Hospital of Liège, University of Liège, 4000 Liège, Belgium
Author contributions: Le Dinh H performed the literature review and wrote the manuscript; de Roover A, Kaba A, Lauwick S, Joris J, Delwaide J, Honoré P and Meurisse M constitute the team involved in the care of the liver transplant patients and they reviewed and commented the manuscript; Detry O supervised the review.
Correspondence to: Olivier Detry, Professor, Department of Abdominal Surgery and Transplantation, University Hospital of Liège, University of Liège, Sart Tilman B35, 4000 Liège, Belgium. oli.detry@chu.ulg.ac.be
Telephone: +32-4-3667645 Fax: +32-4-3667069
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Abstract

The renewed interest in donation after cardio-circulatory death (DCD) started in the 1990s following the limited success of the transplant community to expand the donation after brain-death (DBD) organ supply and following the request of potential DCD families. Since then, DCD organ procurement and transplantation activities have rapidly expanded, particularly for non-vital organs, like kidneys. In liver transplantation (LT), DCD donors are a valuable organ source that helps to decrease the mortality rate on the waiting lists and to increase the availability of organs for transplantation despite a higher risk of early graft dysfunction, more frequent vascular and ischemia-type biliary lesions, higher rates of re-listing and re-transplantation and lower graft survival, which are obviously due to the inevitable warm ischemia occurring during the declaration of death and organ retrieval process. Experimental strategies intervening in both donors and recipients at different phases of the transplantation process have focused on the attenuation of ischemia-reperfusion injury and already gained encouraging results, and some of them have found their way from pre-clinical success into clinical reality. The future of DCD-LT is promising. Concerted efforts should concentrate on the identification of suitable donors (probably Maastricht category Ⅲ DCD donors), better donor and recipient matching (high risk donors to low risk recipients), use of advanced organ preservation techniques (oxygenated hypothermic machine perfusion, normothermic machine perfusion, venous systemic oxygen persufflation), and pharmacological modulation (probably a multi-factorial biologic modulation strategy) so that DCD liver allografts could be safely utilized and attain equivalent results as DBD-LT.

Key words: Non-heart-beating donation; Complication; Bile duct; Allocation; Ischemia; Ischemia-reperfusion injury; Liver disease

Peer reviewers: Bijan Eghtesad, Associate Professor, Department of General Surgery, Cleveland Clinic Foundation, 9500 Euclid Avenue, Cleveland, OH 44195, United States; Tokihiko Sawada, Associate Professor, Second Department of Surgery, Dokkyo University School of Medicine, Kitakobayashi 880, Mibu, Shimotsuga, Tochigi 321-0293, Japan; Philip Rosenthal, Professor, Pediatrics, UCSF, 500 parnassus Avenue, San Francisco, CA 94143-0136, United States

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INTRODUCTION

The first human liver transplantations (LT) were performed from donation after cardio-circulatory death (DCD) in the 1960s[1-4]. DCD-LT was nonetheless almost universally abandoned in the following two decades, given the well-recognized Harvard brain-dead concept in 1968 and given the better results of LT originating from donation after brain death (DBD)[5]. In 1983, LT was approved as a therapeutic modality for end-stage liver diseases after a long period considered as an experimental procedure. The renewed interest in DCD donors started in the 1990s following the limited success of the transplant community to expand the DBD organ supply and following the request of potential DCD families.

If DCD kidneys are increasingly accepted around the world[6], the use of DCD livers remains limited in experienced transplant centers due to higher risks of primary graft dysfunction and biliary complications as well as a lack of a reliable viability testing prior to liver implantation. However the number of DCD-LT increased rapidly over the past decade. In the United States, 276 DCD liver transplants were performed in 2008 compared to only 23 cases in 1999, making up 5% of the deceased donor (DD) liver transplants[7-10]. The same trend was observed in the United Kingdom[11-13], Spain[14], Netherlands[15] and Belgium[15,16]. Netherlands had the highest rate of DCD- over DD-LT in the world (22.5% in 2008)[15]. France has just initiated its DCD-LT program since 2010[17]. In Japan, although DCD donors were the essential DD source, its use was reserved mainly for kidney, pancreas and islet transplantation[18]. Using a mathematical model to analyze the potential impact of a DCD policy on LT programs, Chaib *et al*[19] reported if 1%, 5% and 10% of deceased individuals became DCD donors, there would be 8%, 27% and 37% relative reductions in the size of waiting list, respectively. The use of DCD livers could increase the supply of transplants by 53%[20]. Centers with active DCD-LT programs usually reported 4%-10% rates of LT from the DCD source[21]. The potential impact of DCD use on the DBD availability is also a controversial issue. Controlled DCD programs might negatively influenced DBD activity in Belgium, Netherlands and United Kingdom while uncontrolled DCD donors seemed to be a clear additional source of organs for transplantation in France and Spain[22].

Most countries use Maastricht-category-3 DCD donors for LT, except France and Spain, where categories 1 and 2 are exclusively used due to legal interdiction of discontinuation of therapy in irreversibly brain-injured individuals[17,23,24]. German law prohibits any DCD organ procurement and transplant activity. In Italy, death of a human being must be declared 20 min after cardiac arrest using continuous electrocardiography. The procedure therefore will enable, at best, retrieval of only a few marginal kidneys and some tissues, and will not be helpful for patients on LT waiting lists[25]. This article is aimed at reviewing mono- and multi-centric DCD-LT outcomes, experimental strategies on animal models to optimize the utilization of this donor source and its future development.

DIFFERENCES BETWEEN DCD AND DBD DONORS PERTINENT TO LT OUTCOMES

Generally results of DCD-LT are inferior to those from DBD-LT with regard to both short-and long-term graft and patient survival as well as post-transplant morbidity. Expected DCD-LT outcomes could be explained by inherent differences between DCD and DBD donors in circumstances of death, warm ischemia time (WIT) and donor cause of death. Consequently, a different strategy of DCD use in terms of logistics of organ retrieval and preservation, allocation and recipient selection appears necessary to guarantee acceptable results. These differences will be briefly discussed prior to considering results of DCD-LT in detail.

Circumstances of death and consequent warm ischemia time

In DCD, donor death is diagnosed on the basis of irreversible cessation of cardio-pulmonary function instead of conventional neurologic criteria. As a result, organs from DCD donors are subjected to a period of hypotension, hypoxia and a circulation prior to organ procurement and this WIT adversely affects tissue viability and graft function after transplantation[26]. An international classification of DCD donors into 4 categories was first proposed in 1995 and widely accepted up to now[27]. New DCD categories have been recently suggested in Spain[28,29], Italy[30] and Belgium[31]. The length of WIT varies greatly according to the type of DCD process. It is longest among uncontrolled categories 1 and 2 (usually 90-120 min) and shorter among controlled categories 3 and 4 DCD donors (usually 20-30 min). In brain death, issues related to donor warm ischemia are eliminated because DBD donors have an effective natural organ perfusion and a potentially well-preserved organ function and WIT is thus nearly equal to zero.

However, WIT is heterogeneously defined among authors[32]. In the controlled DCD context, the commonest definition is the time interval between withdrawal of both ventilator and cardiac support to start of cold flushing of the organ[33,34]. This definition includes the no-touch period and the time of death declaration and is proposed to have two phases (withdrawal and acirculatory phases). Other authors used a blood pressure (BP) or oxygen saturation threshold below which would be defined as the beginning of true WIT (systolic or mean BP < 35-60 mmHg, oxygen saturation < 25%-70% or unreadable)[35-42]. de Vera *et al*[34] did not use a BP threshold to define the start of WIT because tissues are still hypoxic in a DCD donor who maintains a BP but has ceased to ventilate. It is unknown at what BP or oxygen saturation the liver parenchyma and biliary system undergo irrecoverable injury[43]. The first international Non-Heart Beating Donor workshop in Maastricht in 1995

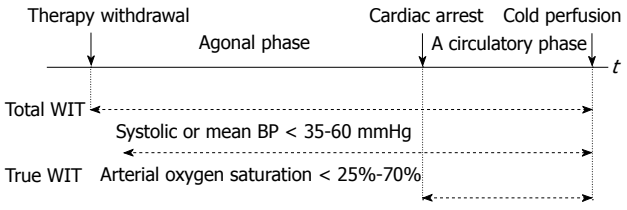

Figure 1 Different ways of warm ischemia time definition in the controlled donation after cardio-circulatory death setting (see text for more details). True warm ischemia time (WIT) is also called complete or functional WIT; Total WIT is also called overall WIT; Agonal phase is also called withdrawal phase. BP: Blood pressure; *t*: Time.

suggested WIT should be calculated from the moment of cardiac arrest until the start of hypothermic flush-out[44]. This definition may be useful for consistency but is inaccurate at the cellular level. Hypoxia starts when the blood flow or oxygenation no longer meets cellular metabolic needs[37]. The start of WIT may be chosen prior to asystole, and the end of WIT may be at or after aortic flushing[45]. Apparently a well-accepted definition of donor organ ischemic times is needed to standardize nomenclature and allow accurate comparisons of individual DCD studies[46,47] (Figure 1).

In transplant practice, WIT should be minimized as much as possible. For controlled DCD donors, the possibility to predict whether a potential donor will or will not expire in a time frame consistent with donation is extremely important, because prolonged time to asystole, likely resulting in suboptimal organ perfusion, is a common reason for non procurement of DCD grafts[48,49]. Time between therapy withdrawal and cardiac arrest usually does not exceed 1 h in most DCD donors. However, if a DCD donor has a period of relatively hemodynamic stability after life-support withdrawal, this period may be extended beyond 1 h without additional warm injury to the organs[50]. Some authors emphasized during the withdrawal phase, time to a systolic BP < 50 mmHg should be < 30 min[20] and the hypotensive period (mean BP < 50 mmHg) < 15 min[51]. Manara *et al*[52] proposed the so-called functional WIT, which is measured from the donor's systolic BP < 50 mmHg, the arterial oxygen saturation < 70%, or both, to the start of cold perfusion, should not exceed 30 min and may be limited to 20 min in suboptimal donors. Several factors have been identified as predictors of rapid death following treatment withdrawal and include the DCD tool of University of Wisconsin[53], donor Glasgow coma scale, inotropic use, BP at treatment discontinuation, high FiO_2 and mode of ventilation[54,55]. Withdrawal of therapy is preferably occurred in the operating room with a donor surgical team immediately available. Prior to cessation of the ventilator and organ perfusion support, the donor may be already prepared and draped, and the surgical instruments, preservation solution and tubing are set up to facilitate rapid organ recovery. The super rapid recovery technique is preferable and organs may be removed *en bloc*[39,50]. For uncontrolled donors, *in vivo* organ preservation techniques, like in-situ intravascular cooling using a double balloon and triple lumen catheter or hypo- and normothermic cardiopulmonary bypass with extracorporeal membrane oxygenation (ECMO), should be employed. With regard to the logistic organization, two frequently mentioned initiatives are the "Maastricht's box" and the "Madrid's rapid identification and response system"[6].

Donor cause of death

DCD donors do not experience the brain dead process. Brain death provokes a cascade of changes in hemodynamics, hormones, and immune response, which negatively affect donor organ viability and transplant outcomes[56,57]. Hemodynamic instability may have deleterious effects on liver function, although the liver has a high tolerance to marked hypotension and a large physiological reserve. Only a few histological changes were observed in the liver both on light and electron microscopic examination during the brain dead process[58,59]. The most important changes are the increased liver immunogenicity with subsequent increased host alloresponsiveness and the occurrence of apoptosis of hepatocytes[60]. Clinical findings in livers from DBD donors revealed significantly higher leukocyte infiltrates, up-regulation of adhesion molecules [intercellular adhesion molecule (ICAM), vascular cell adhesion molecule] and pro-inflammatory cytokines [interleukin-6 (IL-6), IL-10, IL-1β, interferon γ and tumor necrosis factor-alpha (TNF-α)], along with an increased expression of major histo-compatibility complex-Ⅱ relative to livers from living donors[61,62]. The peak time of cytokine expression and cell infiltration is during brain death and organ procurement but not after reperfusion[61]. These changes may amplify ischemia-reperfusion injury (IRI) during the transplant procedure and accelerate graft rejection after transplant[63]. In reality, donor brain-death mechanisms are quite varied and large differences may exist in the degree of impaired organ quality and transplant outcomes. The impact of donor cause of death on transplant outcomes has been recently confirmed in a United Network for Organ Sharing (UNOS) registry analysis, in which the cerebro-vascular accident presented as a predictor of worse graft survival across all organs relative to other donor modes of death[64].

Uncontrolled DCD donors whose cause of death is usually other than neurologic do not undergo the process of brain death, while most controlled DCD donors have sustained irreversible cerebral injuries. As a result, organs from controlled DCD donors are likely to suffer more from the harmful immunologic and inflammatory effects of acute brain injury than those from uncontrolled DCD donors[65].

Allocation policy

It is reported that organs that have already subjected to warm ischemic injury have an increased susceptibility to damage during cold storage[66]. The incidence of primary non-function (PNF) was 2.5 times less in patients with

Table 1 Risk classification for donation after cardio-circulatory death donors and donation after cardio-circulatory death-liver transplantations recipients

Authors		Donors	Recipients
Mateo *et al*[74]	Low risk	Both WIT ⩽ 30 min and CIT ⩽ 10 h	RCRR ⩽ 1.5
	High risk	WIT > 30 min and/or CIT > 10 h	RCRR > 1.5 Re-transplantation and/or On life-support and/or A combination of ⩾ 3 risk factors: Hospitalization or in an intensive care unit Serum creatinine > 2 mg/dL On dialysis Age > 60 yr
Lee *et al*[32]	Low risk	Donors with no identified donor risk factors	Recipients with no identified recipient risk factors
	High risk	Donors with at least one identified donor risk factor: Donor age > 45 yr WIT > 15 min CIT > 10 h	Recipients with at least one identified recipient risk factor: Previous transplantation Life support at transplantation
de Vera *et al*[34]	Low risk	-	MELD scores ⩽ 30
	High risk	-	MELD scores > 30 On life support (mechanical ventilation, hemodialysis)

RCRR: Recipient cumulative relative risk; WIT: Warm ischemia time; CIT: Cold ischemic time; MELD: Model for end-stage liver disease.

cold ischemia time (CIT) ⩽ 8 h *vs* those with CIT > 8 h (5% *vs* 13%)[34]. The incidence of graft failure within 60 d of transplantation was 10.8% if CIT < 8 h and substantially increased to 30.4% and 58.3% if CIT > 8 h and > 12 h, respectively[67]. Proper and rapid allocation of DCD livers thus appears pivotal to minimize CIT. One-year graft survival of DCD livers shared regionally was less good than those shared locally (67% *vs* 77%)[68] and the relative risk of graft failure from nationally shared DCD livers was 31% higher than locally or regionally shared ones[69]. Thus a policy to favor local use of DCD livers seems reasonable[67,68]. However, parallel (backup) offers should also be made to expedite organ placement[33]. The exchange of DCD livers between transplant centers has been successfully done but requires a more efficient and rapid referral system due to a lower tolerance of these allografts to cold storage[70].

Regarding recipient selection criteria, DCD livers could be routinely discussed and offered to all recipients on the waiting list[20,70,71] or selectively reserved to uncomplicated cases to ensure short CIT (by avoiding cases with extensive history of abdominal surgery or portal-vein thrombosis)[20,35]. An expected long surgical procedure exceeding 8 h of CIT, logistical reasons for an extended CIT, combined organ transplantation, recipients with high Model for End-Stage Liver Disease (MELD) scores or a large age difference between donors and recipients could all result in the refusal of a DCD liver[51]. Patients with stable cholestatic liver disease or re-transplantation were also excluded from DCD programs because of problems related to the quality of life in primary biliary cirrhosis and to the fear that pre-existent warm ischemic biliary damage could trigger the recurrence of primary sclerosing cholangitis[72]. Using DCD livers in re-transplanted patients might increase the CIT associated with a difficult hepatectomy. Recently LaMattina has demonstrated the feasibility of simultaneous liver and kidney (SLK) transplantation using DCD donors and shown short-term results comparable to those of SLK transplantation using DBD donors, making it a valid approach to safely expanding the donor organ pool for patients with end-stage liver and kidney disease[73].

It is still controversial whether it is better to transplant such grafts into healthy or sicker recipients (i.e., according to the recipient liver disease severity). UNOS database reviews advocated utilizing DCD livers in "low-risk" recipients[32,67,74]. de Vera *et al*[34] also observed better graft survival when DCD livers were utilized in patients with MELD scores ⩽ 30, but simultaneously could demonstrate that "sicker", high-risk recipients (at MELD scores > 30 or on organ-perfusion support, like mechanic ventilation or hemodialysis) had a greater patient and graft survival benefit from the transplantation of DCD livers compared to patients who are not as critically ill. Risk classification for DCD donors and DCD-LT recipients is summarized in Table 1. Other groups of patients that may have a true survival benefit from DCD-LT include MELD "disadvantaged" patients (hepato-cellular carcinoma patients beyond the Milan criteria or who are listed in areas with long waiting times, patients with low MELD scores that do not adequately reflect their level of illness and their critical need for a transplant)[34,72].

Studies about the effect of DCD liver grafts on hepatitis-C virus positive (HCV+) recipients transplant outcomes were inconsistent. Nguyen *et al*[45] and recently Hernandez-Alejandro *et al*[75] found a negative effect of HCV on DCD livers, but a formal contraindication for the use of DCD liver allografts in HCV+ recipients was not justified except for older donors. In fact, while single-center series reported no significant difference in graft and patient survival rates of HCV+ recipients and graft loss from HCV recurrence between DCD and DBD groups[20,34,76,77], as well as no deleterious effects of DCD liver grafts on the disease progression (fibrosis)

in comparison with DBD liver grafts in HCV+ recipients[77], the most recent UNOS registry data showed inferior graft survival but similar patient survival of HCV+ recipients with DCD donors compared to ones with DBD donors. Furthermore, DCD livers on HCV disease do not fare worse than DCD livers on non-HCV disease. DCD livers thus appeared to be important source of LT for HCV patients[78]. Split livers from DCD donors have also been reported in recent years with acceptable results[79,80].

TRANSPLANT OUTCOMES

Currently one-year patient survival after DBD-LT and to a certain extent after controlled DCD-LT is about 85%-90% in comparison to 60% in the early eighties and around 30% in the early days of LT and at 5 years post-transplant patient survival rate remains over 70%. Medical progress over the past 40 years in the field of organ preservation, surgical techniques, immunosuppressive drugs, treatment of post-transplant complications and organ allocation has permitted DCD to become reality in the modern era. Although there are concerns about the quality of such organs, with evidence that a prolonged WIT causes a raised incidence of PNF and biliary complications as well as suboptimal graft and patient survival when compared to DBD livers, DCD livers may be life-saving for those who would die waiting for a DBD liver[68] and do increase the number of organs available for LT. With careful donor/recipient selection and matching, minimization of ischemia and good post-operative care, acceptable results can be achieved. Essential results of most important publications in the last decade in DCD-LT are presented in Tables 2-4.

PNF

PNF is usually defined as unrecoverable hepato-cellular dysfunction leading to patient death or re-transplantation within the first week post-transplant after excluding other causes of graft failure such as vascular thrombosis, biliary complications, rejection or recurrent disease[81-84]. Initial studies using uncontrolled DCD donors reported a rate of PNF as high as 50%[85]. Currently only a few transplant centers in the world (like Spain, France) used this kind of donors because of aforementioned reasons. By using different *in vivo* organ preservation methods to maintain DCD donors and by strictly applying donor selection criteria, authors in Madrid[71], Barcelona[29] and La Coruña[86-89] could obtain promising results from Maastricht category I and II donors with a PNF rate of 10%-25%. The discard rate nevertheless was high up to 50%-75%[29,71]. In controlled DCD donors, the PNF rates are 0% to 12%. Matched analysis[34,72] and registry data[67,68] showed a higher rate of PNF in controlled DCD than DBD donors, although no difference was found in most comparative studies[20,43,90,91] except one[92]. The increased risk of PNF in DCD-LT recipients was also confirmed in a recent meta-analysis (odds ratio = 3.6, 95% CI: 2.1-6.4)[93]. Case-series reports of controlled DCD-LT also had a rate of PNF between 0% and 10%[42,70,94-97].

PNF is the consequence of severe IRI with the initial period of warm ischemia playing a crucial role. Experimental evidence supported that donor WIT should be less than 30 min to minimize PNF[98]. This warm ischemia (WI) period increases graft susceptibility to damage during cold preservation and CIT was a main contributing factor to PNF[34,67]; therefore, both periods of ischemia must be kept to a minimum. Many laboratory tests have been developed both in animal models and in human to predict the probability of occurrence of PNF post-transplant, but none is yet clinically efficient[99]. Recently Dahaba *et al*[100] proposed bispectral index monitoring as an early intra-operative indicator of early graft dysfunction.

Biliary complications

Since the introduction of LT up to now, biliary complications are always regarded as the "Achilles heel" and a major cause of morbidity and graft failure in patients after LT[101]. The most common biliary complications are bile leakage and bile duct stricture[102,103]. Strictures involving the donor bile duct (> 1 cm above the biliary anastomosis) and requiring endoscopic or radiological dilatation/stenting or surgery in the face of a patent, non-stenotic hepatic artery was referred to as ischemic-type biliary lesions (ITBL), based on the radiologic resemblance of those occurring after hepatic artery thrombosis (HAT)[51,91,103].

Abt *et al*[104] first mentioned the significantly higher incidences of overall biliary complications as well as ITBL in DCD-LT recipients, the finding which was later confirmed in both matched[34,72] and comparative[20,43,51,89,91,104] studies except series of Fujita *et al*[105] and Manzarbeitia *et al*[106]. The rates of overall biliary complications and ITBL were 10.5%- 53% and 8.3%-38%, respectively in DCD-LT compared to 8.3%-22% and 0%-8%, respectively in DBD-LT. Especially Jiménez-Galanes *et al*[71] reported only a 5% incidence of ITBL in their patients receiving livers from uncontrolled DCD donors under normothermic ECMO. A recent meta-analysis revealed that DCD recipients had a 2.4 times increased odds of biliary complications (95% CI: 1.8-3.4) and a 10.8 times increased odds of ITBL (95% CI: 4.8-24.2) *vs* DBD recipients. In average, biliary complications were present in 29% of DCD compared with 17% of DBD recipients and ITBL in 16% of DCD *vs* 3% of DBD recipients[93].

Furthermore DCD recipients who developed ITBL experienced a fairly rapid clinical deterioration, characterized by a relatively short mean time from transplant to first endoscopic retrograde percutaneous cholangiopancreatography (ERCP), from first ERCP to relisting and from relisting to re-transplantation (within 180 d)[36,69]. ITBL results in re-operation, multiple endoscopic and percutaneous biliary interventions, re-transplantation and even patient death with markedly increased medical care costs[107]. The relative risk (RR) of developing graft loss with ITBL formation was 3.02 (95% CI: 1.9-5.3)

Table 2 Results of donation after cardio-circulatory death-liver transplantations in single-center studies

Authors study period	Transplant center	Publication year	Patient number and Maastricht category	WIT (min)	CIT (min)	Mean follow-up	PNF %	Major biliary complications %	ITBL %	HAT %	HAS %	Rejection %	Retransplantation %	Graft survival %				Patient survival %			
														1 yr	3 yr	5 yr	10 yr	1 yr	3 yr	5 yr	10 yr
Casavilla *et al*[85]	Pittsburgh,	1995	6 DCD_4	37	10.6 h	-	50[1]	-	-	16.6	-	-	83.3	17	-	-	-	67	-	-	-
1989-1993	United States		6 DCDc	23.8	11 h	-	0[1]	-	-	33.3	-	-	33.3	50	-	-	-	50	-	-	-
Otero *et al*[87]	Madrid, Spain	2003	20 DCD_2	10	647	> 2 yr	25[1]	30[1]	-	0	-	27	25	80	-	-	-	80	-	-	-
1995-2000			40 DBD	8	405	-	3[1]	8[1]	-	-	-	34	5	55	-	-	-	83	-	-	-
Quintela *et al*[86]	Spain	2005	9 DCD_2 + 1 DCD_4	80	561	-	10	-	-	-	-	-	10	100	-	-	-	100	-	-	-
1995-2004																					
Suárez *et al*[89]	Spain	2008	27 DCD_2	13	635	> 3 mo	18[1]	41.7[1]	25.0[1]	3.6	-	17.4	-	-	-	49[1]	-	-	-	62	-
1994-2005			471 DBD	7	-	-	3[1]	16.8[1]	2.3[1]	3.1	-	28.6	-	-	-	68[1]	-	-	-	74	-
Fondevila *et al*[29]	Barcelona, Spain	2007	10 DCD_1	-	399	-	10	10	-	10	-	-	20	50	-	-	-	70	-	-	-
2002-2006			20 DBD	-	-	-	0	0	-	5	-	-	5	75	-	-	-	80	-	-	-
Jiménez-Galanes *et al*[71]	Madrid, Spain	2009	20 DCD_2	12	432	360 d	10	5	50	0	-	-	1[1]	80	-	-	-	85.5	-	-	-
2006-2008			40 DBD	6	409	-	2.5	-	-	-	-	-	50[1]	87.5	-	-	-	87.5	-	-	-
Pine *et al*[72]	St. James, London,	2009	39 DCDc	13.4	352	2.5 yr	5.1	33.3[1]	20.5[1]	2.6	12.8[1]	20.5	7.6[1]	79.5[1]	63.6[1]	-	-	80[1]	68.2[1]	-	-
2002-2008	United Kingdom		39 DBD	-	593	6.6 yr	-	10.2[1]	-	5.1	-	23.1	2.5[1]	97.4[1]	97.4[1]	-	-	100[1]	1[1]	-	-
de Vera *et al*[34]	Pittsburgh,	2009	141 DCDc	19.8	657	-	12[1]	25[1]	16.3[1]	66	-	-	18[1]	69[1]	-	56[1]	44[1]	79	-	70	57
1993-2007	United States		282 DBD	-	636	-	3[1]	13[1]	< 1[1]	-	-	-	7[1]	82[1]	-	73[1]	63[1]	85	-	76	64
Yamamoto *et al*[90]	Stockholm, Sweden	2010	24 DCDc	6	7 h	> 20 yr	8.3	37.5[1]	-	33.3[1]	-	70.8	-	54.2	-	37.5	37.5	61.9	-	42.9	42.9
1984-1988			16 DBD	-	6.8 h	> 20 yr	18.7	6.3[1]	-	-	-	56.2	-	43.8	-	37.5	37.5	63.6	-	-	54.5
Fujita *et al*[105]	Gainesville, Floria,	2007	24 DCDc	12.8	7.6 h	-	2.8	25	12.5	8.3	-	39.1	20.8	69.1	58.6	-	-	86.8	81.7	-	-
1990-2006	United States		1209 DBD	-	8.1 h	-	-	20.5	-	4.1	-	-	9.4	78.7	70.2	-	-	84	76	-	-
Foley *et al*[91]	Wisconsin,	2005	36 DCDc	17.8	8.2 h	3 yr	5.5	33[1]	13.8	5.5	16.6[1]	61	19.4[1]	67[1]	56[1]	-	-	80[1]	68[1]	-	-
1993-2002	United States		553 DBD	-	8.3 h	4.6 yr	1.3	10[1]	8	11.8	5.4[1]	56	7[1]	86[1]	80[1]	-	-	91[1]	84[1]	-	-
Manzarbeitia *et al*[106]	Philadelphia,	2004	19 DCDc	19.6	574	1000 d	5.2	10.5	-	-	-	-	10.5	-	-	-	-	89.5	-	-	-
1995-2002	United States		311 DBD	-	557	-	-	13.8	-	-	-	-	8.7	-	-	-	-	84.2	-	-	-
Abt *et al*[104]	Pennsylvania,	2003	15 DCDc	20.4	366	819 d	6.7	33.3[1]	26.7[1]	3.2	-	20	6.6[1]	71.8	71.8	-	-	79	79	-	-
1996-2001	United States		221 DBD	-	464	690 d	3.6	9.5[1]	2.3[1]	-	-	21.3	3.6[1]	85.4	73.9	-	-	90.9	77.7	-	-
Nguyen *et al*[45]	Mayo Clinic, Floria,	2009	19 DCDc	16	6.7 h	> 4.5 yr	5.3	26.3	10.5	0	5.3	5.3[1]	15.8	73.7	68.4	63.2	-	89.5	89.5	89.5	-
1998-2001	United States		234 ECD	-	7.1 h	-	4.7	22.6	-	-	-	33.3[1]	8.5[1]	-	-	-	-	85	78.6	72.3	-
			214 SCD	-	7.5 h	-	1.7	15.9	-	-	-	33.2[1]	19.6[1]	-	-	-	-	84.3	80.7	76.5	-

DCDc: Controlled donors after cardiac death; DCD_1, DCD_2 and DCD_4: Maastricht category-1, category-2 and category-4 DCD donors; DBD: Donors after brain death; SCD: Standard criteria donors; ECD: Extended criteria donors; WIT: Warm ischemia time; CIT: Cold ischemia time; PNF: Primary non-function; ITBL: Ischemic-type biliary lesions; HAT: Early hepatic artery thrombosis; HAS: Early hepatic artery stenosis. Major symptomatic biliary complications include biliary leak, anastomotic and non-anastomotic stenosis. [1]Numbers denote the statistically significant difference between groups.

and graft survival was significantly decreased in patients with non-anastomotic strictures, compared to patients without it[89]. Up to 50% of all occurrences of ITBL lead to death and/or re-transplantation[108].

ITBL is usually a reflection of severe IRI in relation to various factors. In animal models, irreversible biliary tract damage has been observed after 40 min of cardiac arrest although hepato-cellular function could be preserved[109]. Clinical observations showed that total WIT > 30 min and chaotic donor physiology before asystole may increase the risk of post-transplant biliary stricture[33,110]. The mechanism could come from the stasis of blood and clot formation in the peri-biliary micro-circulation whose blood is solely supplied by the hepatic artery[96]. Many multivariate analysis recognized DCD liver grafts as an independent risk factor for the appearance of ITBL (RR = 47.1)[51,89].

Table 3 Results of donation after cardio-circulatory death- liver transplantations in single-center studies

Authors study period	Transplant center	Publication year	Patient number and Maastricht category	WIT (min)	CIT (min)	Mean follow-up	PNF %	Major biliary complications %	ITBL %	HAT %	HAS %	Rejection %	Retransplantation %	Graft survival %			Patient survival %		
														1 yr	3 yr	5 yr	1 yr	3 yr	5 yr
Grewal *et al*[20]	Mayo Clinic, Floria,	2009	108 DCDc	22.3	6.3 h	-	3.7	-	8.3[1]	0.9	-	-	14.8	79.3	74.5	71	91.5	88.1	88.1
1998-2006	United States		1328 DBD	-	7.1 h	-	1.4	-	1.9[1]	1.7	-	-	9.3	81.6	74.7	69.1	87.3	81.1	77.2
Kaczmarek *et al*[41]		2007	11 DCDc	34.6	7.6 h	> 14 mo	0	45.4[1]	1[1]	0	-	-	9.1[1]	-	-	-	-	-	-
1999-2006			164 DBD	-	-	-	-	16.4[1]	8.2[1]	-	-	-	0[1]	-	-	-	-	-	-
Dubbeld *et al*[51]		2010	55 DCDc	16.5	456	-	2	28[1]	24[1]	74.7	-	-	18	74	68	-	85	80	-
2001-2006			471 DBD	-	515	-	1.5	8.3[1]	7.9[1]	-	-	-	10.4	80.4	74.5	-	86.3	80.8	-
Chan *et al*[43]	Seattle, United States	2008	51 DCDc	-	-	3 yr	0	23.5[1]	13.7[1]	4.8	-	-	9.8	79	79	-	83	83	-
2003-2006			334 DBD	-		-	3.3	8.9[1]	1.2[1]	-	-	-	-	85	77	-	88	78	-
Skaro *et al*[36]	Chicago, United States	2009	32 DCDc	15.8	5.5 h	-	3	53[1]	38[1]	93	-	-	2[1]	61[1]	53[1]	-	74	74	-
2003-2008			237 DBD	-	5.2 h	-	1	22[1]	2[1]	-	-	-	27[1]	85[1]	74[1]	-	90	81	-
Jay *et al*[107]	Chicago, United States	2010	28 DCDc	16.5	5.7 h	1.8 yr	3.6	57.7[1]	44[1]	10.7	7.1	-	21.4[1]	60[1]	50[1]	-	70[1]	70[1]	-
2004-2008			198 DBD	-	5.3 h	-	0.5	21[1]	1.6[1]	3	6.1	-	7.1[1]	89[1]	78[1]	-	96[1]	93[1]	-
Dezza *et al*[92]	Ghent, Belgium	2007	13 DCDc	10	6.16 h	163 d	8[1]	-	23.1[1]	-	-	-	31[1]	54[1]	-	-	62[1]	-	-
2003 -2006			98 DBD	-	9.14 h	603 d	1[1]	-	-	-	-	-	12[1]	79[1]	-	-	86[1]	-	-
Maheshwari *et al*[95]	Johns Hopkins Balti-		20 DCDc	33	8.7 h	-	5	60	50	5	-	-	20	62	62	30	78	78	40
1997-2006	more, United States																		
Muiesan *et al*[70]	London,	2005	31 DCDc	14.7	8.6 h	-	3.1	9.4	0	3.1	-	28.1	3.1	86.5	-	-	89.6	-	-
2001-2004	United Kingdom																		
Abou Abbass *et al*[97]	United States	2010	26 DCDc	39	5.3 h	-	0	46	15.4	11.5	7.7	26.9	23	77	-	-	92	-	-
2004-2008																			
Detry *et al*[94]		2010	58 DCDc	25	451	-	3.4	38	32.7	3.4	3.4	-	13.8	72.4	48.8	-	83.3	66.9	-
2003-2007																			
Hernandez-Alejandro *et al*[42]	London,	2010	10 DCDc	54.7	5.8 h	-	10	10	0	0	0	-	10	-	-	-	-	-	-
2006-2007	United Kingdom																		
Hashimoto *et al*[96]	United States	2010	22 DCDc	21	422	-	4.5	27	9	0	0	-	9	81	81	-	-	-	-
2005-2009																			

DCDc: Controlled donors after cardiac death; DBD: Donors after brain death; SCD: Standard criteria donors; ECD: Extended criteria donors; WIT: Warm ischemia time; CIT: Cold ischemia time; PNF: Primary non-function; ITBL: Ischemic-type biliary lesions; HAT: Early hepatic artery thrombosis; HAS: Early hepatic artery stenosis. Major symptomatic biliary complications include biliary leak, anastomotic and non-anastomotic stenosis. [1]Numbers denote the statistically significant difference between groups.

Biliary epithelium is also known to be sensitive to cold preservation-reperfusion injury and the correlation between the incidence of ITBL and the duration of cold ischemia has been well documented. Li *et al*[111] demonstrated that the rate of ITBL is significantly increased in livers with increased preservation injury, as reflected by post-transplant peaks in serum transaminases. Other variables implicating in the mechanisms of ITBL may include injury of the peri-biliary vascular plexus, bile salt toxicity and potential immunological etiologies (ABO incompatibility, liver diseases with autoimmune component like autoimmune hepatitis and primary sclerosing cholangitis)[102]. Chan *et al*[43] found donor age > 50 years, donor weight ≥ 100 kg and total ischemia time ≥ 9 h were predictive for the development of ITBL. Patients who underwent LT from DCD donors > 60 years had a markedly high rate of biliary complications (67%), with a RR of 5.6 (95% CI: 0.98-32.2)[34].

Due to serious consequences of ITBL on the patient's quality of life and healthcare cost, preventive measures seem to play a pivotal role in the safe expansion of DCD liver use. Attempts to minimize biliary duct damage may include the use of normothermic ECMO for donor maintenance[29,71,112] and machine perfusion for liver grafts, choice of preservation solutions [histidine-tryptophan-ketoglutarate (HTK) *vs* University of Wisconsin][113-117], use of anticoagulation and thrombolytic agents[96], extensive

Table 4 Results of donation after cardio-circulatory death-liver transplantations in United Network for Organ Sharing data base registry

Authors and study period	Publication year	Patient number and Maastricht category	WIT (min)	CIT (h)	PNF (%)	Retransplantation (%)	Graft survival %			Patient survival %		
							1 yr	3 yr	5 yr	1 yr	3 yr	5 yr
Abt *et al*[67]	2004	144 DCD	12.7	8.1	11.8[1]	13.9[1]	70.2[1]	63.3[1]	-	79.7	72.1	-
1993-2001		26 856 DBD		8.9	6.4[1]	8.3[1]	80.4[1]	72.1[1]		85	77.4	
Mateo *et al*[74]	2006	367 DCD	15.6	8.3	-	-	71[1]	60[1]	53[1]	-	-	-
1996-2003		33 111 DBD		8.4			80[1]	72[1]	65[1]			
Lee *et al*[32]	2006	874 DCD	15.4	7.9	-	-	72.1[1]	61.8[1]	38.8[1]	82.3[1]	75.9[1]	65.3[1]
1996-2006		43 734 DBD		8.2			80.7[1]	71.9[1]	65.6[1]	85.4[1]	77.5[1]	71.5[1]
Doshi *et al*[68]	2007	345 DCD	-	8.2	6.4[1]	13.0[1]	75	65	-	83	77	-
1998-2004		20 289 young-DBD		8.1	3.9[1]	5.6[1]	83[1]	75[1]		88[1]	80[1]	
		3604 old-DBD		8.2	5.3[1]		76	64		83	73	
Merion *et al*[125]	2006	472 DCD	-	7.9	-	-	70.1[1]	60.5[1]	-	-	-	-
2000-2004		23 598 DBD		8.1			83[1]	75[1]				
Selck *et al*[69]	2008	855 DCD	-	-	-	21.6[1]	73.8[1]	57.6[1]	-	-	-	-
2002-2007		21 089 DBD				8.8[1]	84.4[1]	74.4[1]				
Mathur *et al*[127]	2010	1567 DCD	16.1	7.5	-	13.6	-	-	-	78	64.9	-
2001-2009												

DCD: Donors after cardiac death; DBD: Donors after brain death; WIT: Warm ischemia time; CIT: Cold ischemia time; PNF: Primary non-function. Major symptomatic biliary complications include biliary leak, anastomotic and non-anastomotic stenosis. [1]Numbers denote the statistically significant difference between groups.

irrigation of the donor bile duct and pressure perfusion of the hepatic artery during organ retrieval and/or at back table[113,118,119], early porto-caval shunt to reduce portal hypertension in the recipient, choice of reperfusion techniques (concomitant *vs* sequential reperfusion of portal vein and hepatic artery)[120] and certainly the most important thing is always minimizing warm and cold ischemia period[121].

HAT and stenosis

HAT is a thrombo-embolic occlusion of the hepatic artery that can occur early or late after LT. Most authors used the first 30 d post-transplant as a time point to distinguish between early and late HAT[122]. Early HAT results in fulminant hepatic failure, bile duct necrosis and leaks, relapsing bacteremia and ultimately graft loss and recipient death. The frequencies of early HAT after DCD-LT varied from 0% to 16.6% and did not seem significantly higher than those after DBD-LT in most studies[20,29,34,36,43,51,71,72,89,91,104,105] except Yamamoto *et al*[90] (33.3% *vs* 0%). Risk factors for early HAT have been well analyzed in a recent systemic review[123]. Few detailed studies discussed late HAT.

The incidence of hepatic artery stenosis (HAS) was not consistently found higher in DCD than DBD grafts (12.8%-16.6% *vs* 0%-5.4%)[72,91]. It is possible that hepatic arteries are susceptible to WI during DCD organ retrieval, resulting in subsequent scar and stenosis. Moreover the increased susceptibility of DCD livers to post-operative arterial ischemia might be responsible for more biliary strictures in DCD than DBD recipients with HAS (83% *vs* 37%) as well as shorter time to the development of biliary strictures after HAS in the DCD group[91]. Inadequate surgical technique, vascular trauma by clamps, graft rejection, recurrent hepatic disease might also play a role in the mechanisms for HAS[72,124].

Graft and patient survival

Graft survival is defined as the time from transplantation to either re-transplantation or patient death, with "early" and "late" graft failure occurring within and beyond 1 year post-transplant, respectively[34]. Few studies reported experience with LT from uncontrolled DCD donors. Early results were poor with a PNF rate of 50% and one-year graft survival rate of only 17%[85] leading to a scarce usage of this donor category in the United States. Subsequent series in Spain using advanced *in vivo* organ preservation methods showed promising outcomes with one- and five-year graft survival rates of 50%-80% and 49%, and one- and five-year patient survival rates of 70%-85.5% and 62%, respectively[29,71,89]. LT from controlled DCD donors offered better results although they still appeared inferior to DBD-LT in matched studies[34,72], registry data analysis[32,67-69,74,125] and in some comparative studies[36,91,92]. One-, three-, five- and ten-year graft survival rates were 54%-79.5%, 53%-74.5%, 37.5%-71% and 37.5%-44%, respectively. Patient survival rates at corresponding time points were 61.9%-91.5%, 62.8%-89.5%, 42.9%-89.5% and 42.9%-57%, respectively. Transplant outcomes comparable to those obtained from DBD-LT have been sporadically reported in select centers through careful donor selection and optimization of CIT or through invasive techniques designed to optimize recovery before declaration of death[20,43,51,104].

Significant risk factors for DCD liver graft loss have been identified by multivariate Cox regression technique in both single center studies and large data registry analysis[32,67-69,74,126,127]. Among donor risk factors, age > 50 years, total WIT > 30-35 min, CIT > 6 h, body weight > 100 kg and regional or national liver distribution had deleterious effects on graft survival[32,74,127]. There is a stepwise increase in the relative risk of graft failure among donor age, WIT and CIT[32,127]. Strong recipient determinants of

graft failure include age > 55 years, history of previous transplantation, medical status at transplantation [intensive care unit (ICU) or non-ICU hospitalization, life support, dialysis, renal insufficiency], high MELD score (> 30) and positive HCV serology[32,74,127]. In the DBD-LT model, it has been shown that a single risk factor lessened outcome marginally, however, the additive effect of multiple risk factors in a given donor-recipient pair were disastrous[83]. Grafts with ≥ 3 donor risk factors had significantly lower 1-year post-transplant survival than no or only 1 or 2 risk factors (58.3% *vs* 72.6%, 69.2% and 73.9%, respectively). No grafts with 4 risk factors survived within 1 year[128]. The relative risk of allograft failure from LT utilizing DCD donors was 31%-87% higher than LT utilizing DBD donors[67-69,125,126]. Causes of early graft failure included PNF, biliary complications, HAT and deaths from sepsis/multi-organ failure. Late graft failure was often secondary to chronic rejection and recipient death with a functioning graft.

Although DCD livers may not be as good as DBD ones with potential inferior transplant outcomes, there are subgroups of grafts and recipients that could give favorable results through appropriate graft and recipient matching. Low-risk DCD grafts which are transplanted in low-risk patients lead to comparable graft survival rates with DBD livers. Livers from DCD donors transplanted into high-risk recipients fared poorly independent of the allograft quality[74]. Doshi *et al*[68] showed DCD liver grafts were not inferior to DBD livers from older donors (≥ 60 years). Given the ever increasing demand for LT, DCD livers appear to be a reasonable alternative to increasing use of older or split livers and are a reasonable option when death is imminent[68]. Even if graft or/and patient survival is lower with a DCD liver, it is still better than dying because of turning down a DCD offer and continuing to wait for a DBD liver on these d as the patient' s choice is frequently not between marginal livers (including DCD) and standard livers but between marginal livers and no livers[105]. The benefit of earlier access to LT provided by a DCD graft could outweigh the risks of prolonged waiting for a standard graft[77].

Re-transplantation

DCD recipients more often require re-transplantation. Respectively, 21.6%-42% *vs* 8.8%-16% of DCD and DBD recipients were listed for re-transplantation[36,69]. The re-transplantation rate ranged from 7.6% to 31% in DCD-LT compared to 2.5%-12% in DBD-LT[20,29,34,36,51,67-69,71,72,91,92,106]. DCD livers exhibited a 2.1 times greater risk of graft failure, a 2.5 times greater risk of re-listing, and a 3.2 times greater risk of re-transplantation compared with DBD livers[36]. The majority of re-listing and re-transplantation in the DCD group were a consequence of biliary complications, especially ischemic cholangiography, but not due to an increased incidence of PNF, HAT or technical complications[36,69]. Particularly DCD livers had a temporally different failure pattern within the first year post-transplant that limited access to re-transplantation[36,69]: graft failure was more likely to occur within the first 180 d (18.1% *vs* 11.7%[67], 10.2% *vs* 2.5%[72] and 20.5% *vs* 11.5%[69] of DCD and DBD grafts failed within 60, 90 and 180 d, respectively); at re-transplantation, DCD recipients waited longer and received higher risk allografts; and more DCD recipients remained waiting for re-transplantation with fewer removed for death, clinical deterioration, or improvement. Re-transplantation arouses controversy on medical, economic, and ethical grounds: patient and graft survival rates after a second LT are inferior to those after initial grafting, the procedure is more expensive and in the context of organ shortage, re-transplantation inevitably denies organs to first-time recipients[129].

Utilization of DCD allografts for re-transplantation was rare (2.5% of initial DCD *vs* 3.1% of initial DBD) and outcomes from each group were comparable[69]. The general practice is to avoid re-transplantation with a DCD graft[36]. The use of DCD donors in the setting of re-transplantation resulted in an increased risk of recipient death (hazard ratio = 2.1, 95% CI: 1.2-3.6)[129].

Acute rejection

The acute rejection rate did not differ significantly between DCD- and DBD-LT in most studies (1.9%-29% *vs* 0.6%-34%)[20,72,87,89,104]. Foley *et al*[91] reported a one-year rejection rate of 61% in the DCD group similar to that in the DBD group (56%). There were little data looking at the impact of DCD source on the risk of acute rejection.

EXPERIMENTAL STRATEGIES TO IMPROVE DCD-LT OUTCOMES

The progressively increased DCD liver procurement to solve the shortage of DBD organs and to alleviate the waiting-list mortality has raised many challenges to the transplant community and transplant policy makers[110]. A lot of experimental researches have been performed over the past decade, intervening in both donors and recipients at different phases of the transplantation process, at the aim of tackling some of these challenges and providing a deep insight into IRI mechanisms.

Donor pre-treatment

Various cyto-protective substances have been successfully administered into the donor prior to cardiac arrest for prevention of liver microcirculatory disturbance. Microcirculatory disturbance was the main obstacle to successful DCD-LT, which was due to four major mechanisms: deterioration of sinusoidal endothelial cells (SEC) caused by activated Kupffer cells, sinusoidal narrowing caused by some vasoconstrictors and swollen hepatocytes, leukocyte and platelet adhesion, and hyper-coagulability[130]. Up to now, only *Heparin* and *phentolamin* (an anticoagulative substance and alpha-adrenergic antagonist) are allowed in clinical DCD organ procurement[131], other substances remain in animal models. *Tacrolimus,* besides

its powerful immunosuppression, enabled to prevent liver normothermic IRI by multiple mechanisms[132]. *Milrinone*, a type 3 phosphodiesterase inhibitor, attenuated graft injury caused by warm and cold ischemia *via* an increase in intracellular cAMP levels, protection of SEC, relaxation of hepatic stellate cells, inhibition of platelet aggregation and anti-inflammatory effect[133]. *Lazaroids*, an antioxidant designed to inhibit iron-dependent lipid peroxidation, ameliorated SEC viability *via* antioxidant effects and membrane stabilization[134]. *N-acetylcystein* has a direct effect on oxygen free radicals, but its usage had no effect in both graft viability and lipid peroxidation[135].

Animal studies clearly showed the concept of pharmacological modulation of organ donors before procurement is feasible to improve the viability of marginal grafts. Nevertheless there are no definitive recommendations for the use of these drugs. Application of this method to clinical LT would require management of some practical problems and possible ethical conflicts[136].

Organ preservation

Preservation of DCD livers by hypothermic machine perfusion (HMP) was shown superior to static cold storage (SCS) in many experimental studies[137,138].

Nonetheless a putative drawback of HMP for livers is to induce alterations at the vascular endothelial site, especially if HMP was performed for a long time or under suboptimal conditions[139]. Endoplasmic stress activation promoted cellular apoptosis *via* activation of caspase-12[140,141]. The efficiency of HMP was markedly increased by oxygenation of the perfusate[142]. The concern that high oxygenation might favor the generation of oxygen free radicals, which in turn could impair tissue integrity, was not justified. Several investigators could demonstrate the beneficial effect of oxygenated HMP in reducing the liver expression of pro-inflammatory cytokines (TNF-α, IL-8), adhesion molecules (ICAM-1) and major histocompatibility complex class Ⅱ antigens[143-145]. This benefit will likely be more pronounced in marginal grafts such as elderly, steatotic and DCD livers[144]. Cyto-protective agents can be added into the machine perfusion (MP) solution to ameliorate the efficiency of HMP organ preservation[146].

The positive effects of HMP on warm-ischemically pre-damaged livers were observed even after a brief period of MP, before (pre-conditioning) or after SCS (post-conditioning)[143,147] and therefore it was not necessary to require MP over a full preservation period and helped avoid side-effects of HMP on vascular endothelium[141]. The use of HMP as the initial method for organ preservation followed by secondary SCS during transportation combined the advantage of aerobic resuscitation (i.e., restitution of cellular homeostasis) with an ease of SCS for later surveillance and transportation[141]. Manekeller *et al*[148] showed a post-conditioning of 1 h after SCS can ameliorate the viability of marginal livers. The extension or abbreviation of post-conditioning time seems to have no further beneficial effects[148].

Schön *et al*[66] reported advantages of normothermic machine perfusion (NMP) over SCS in pig DCD-LT models. Livers subjected to 1 h of WI and then cold-stored for 4-24 h were rendered completely nonviable while such livers under 4-24 h of oxygenated NMP recovered function to a viable level[149]. Due to the complexity of the logistics of clinical multi-organ recovery and of the NMP device, a period of cold preservation prior to warm perfusion of the liver is unescapable. A brief period of cold preservation (1 h) prior to NMP could maintain the synthetic and metabolic function but resulted in significant hepatocellular damage, sinusoidal endothelial cell dysfunction and Kupffer cell injury[150]. Once this duration was prolonged to 4 h, NMP completely failed to resuscitate porcine livers[151]. Normothermically perfusing DCD livers throughout the preservation period not only replenished cellular substrate, ameliorating the ischemic injury, but also provided a clear assessment of liver function and therefore could permit the use of severely injured organs with reassurance of function[149,152].

Despite the aforementioned benefits of MP over SCS in liver preservation, only SCS is clinically approved up to now, MP is still in the pre-clinical stage and early clinical studies[153]. Tojimbara *et al*[154] showed the impact of viscosity and temperature of initial flushing solutions on graft function. A low viscosity flushing solution was associated with lower vascular resistance, whereas a warm flush solution prevented cold-induced vasospasm and therefore improved the washout effect of the microcirculation[154]. HTK solution possessing a low viscosity and low potassium is more preferable in the DCD setting. The role of aeration of the cold-stored liver was also clarified. Oxygen provided either by surface diffusion (surface oxygenation) or intravascular diffusion (oxygen persufflation) helps improve the energy status of organs thus leading to earlier recovery. Surface oxygenation was not in use any more due to complicated technique, limited efficiency and risk of oxygen intoxication[155]. Venous systemic oxygen persufflation (VSOP) was shown to improve organ viability during hypothermic storage of the grafts and to be a feasible means for reconditioning of warm-ischemically pre-injured livers from DCD donors[155-158]. Experimentally even a short period of VSOP prior to long-term preservation of the liver by SCS may be sufficient for a relevant improvement of liver integrity upon reperfusion[159]. Gaseous persufflation with carbon monoxide was also tested in a DCD-LT rat model with enhanced liver graft viability[160]. However no additive or synergistic effect was noted when livers were persufflated with a mixture of gaseous oxygen and carbon monoxide[161].

Pharmaceutical interventions during SCS aimed at conditioning marginal organs also increasingly gained attention. Different cyto-protective drugs have been added into the flush and/or preservation solution, like vasodilators (phentolamin, epoprosterol, dopamine)[162,163], anti-coagulants (heparin), fibrinolytic agents (strepto-

kinase)[164], antioxydants (superoxide dismutase, edaravone)[165,166], antibiotics, hormones (glucagon, growth factors)[167]. In the DCD setting, vasodilators, anti-coagulants, thrombolytic agents and antibiotics seem particularly necessary because the organs tend to develop vasospasm, thrombus formation in the microcirculation and the risk of colonic bacterial contamination secondary to translocation of organisms during the WI period[168,169].

Viability testing

Due to serious consequences of transplanting a DCD liver with potentially severe IRI (PNF, re-transplantation or even recipient death), it would be ideal if the viability of such livers could be predicted prior to rather than after transplantation. WIT is not always exactly known and thus cannot be a reliable parameter. Light microscopic examination of biopsy specimens was unable to uniformly predict liver function after transplantation[170]. Monbaliu *et al*[171] showed the extent of parenchyma vacuolation predicted pig liver graft viability before LT. Muiesan *et al*[70] applied the mechanical digestion of liver biopsies with collagenase and assessed the viability of hepatocytes by trypan blue exclusion method. However, the test was not helpful and the decision as to whether to use the liver was generally made on gross appearance, ease of perfusion, degree of steatosis and donor characteristics[172].

Another approach is to evaluate the vascular resistance and enzyme release in the perfusate of HMP livers. Resistance index of the portal vein and hepatic artery showed no utility[173]. Biomarkers of liver cell damage, like transaminases, lactate dehydrogenase and liver fatty acid binding protein, correlated well with WI duration and concomitant hepatocyte damage in pig DCD-LT models[174]. Possible other parameters are the ATP content and redox active iron status of the liver during HMP[175]. During NMP, the assessment of liver viability may be easier because the liver is in a normal metabolic state. Bile production was a good viability indicator besides the measurement of other liver functions (detoxification, metabolism or synthesis)[176]. Recently Liu *et al*[177] has tested the utility of magnetic resonance imaging and proton magnetic resonance spectroscopy to evaluate WI livers without success.

Recipient treatment

Pharmaceutical strategies aimed at modulating IRI mechanisms were also applied successfully in animal recipients and generally did not impose ethical problems as donor pre-treatment. Such protocols without donor pretreatment will be favorable in clinical application. Most studies tested a single agent for a specific target of the IRI process. A multi-factorial approach acting on different pathways of the IRI process have been advocated and remarkably ameliorated transplant outcomes[162].

PERSPECTIVES

The future of DCD-LT is promising. Concerted efforts should concentrate on the identification of suitable donors (probably Maastricht category Ⅲ DCD donors), better donor and recipient matching (high risk donors to low risk recipients), use of advanced organ preservation techniques (oxygenated HMP and NMP, VSOP), and pharmacological modulation (probably a multi-factorial biologic modulation strategy) so that liver procurement and transplantation from DCD donors could be widely expanded and attain equivalent results as DBD-LT.

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REVIEW

Surveillance for gastrointestinal malignancies

Ashish K Tiwari, Heather S Laird-Fick, Ramesh K Wali, Hemant K Roy

Ashish K Tiwari, Heather S Laird-Fick, Department of Internal Medicine, Michigan State University, East Lansing, MI 48824, United States
Ramesh K Wali, Hemant K Roy, Department of Internal Medicine, North Shore University Health System, Evanston, IL 60021, United States
Author contributions: Tiwari AK, Laird-Fick HS, Wali RK and Roy HK contributed equally to this paper.
Correspondence to: Ashish K Tiwari, MD, Department of Internal Medicine, Michigan State University, 138 Service Rd No. A225, East Lansing, MI 48824,
United States. ashish.tiwari@hc.msu.edu
Telephone: +1-312-2088961 Fax: +1-517-4322759
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Abstract

Gastrointestinal (GI) malignancies are notorious for frequently progressing to advanced stages even in the absence of serious symptoms, thus leading to delayed diagnoses and dismal prognoses. Secondary prevention of GI malignancies through early detection and treatment of cancer-precursor/premalignant lesions, therefore, is recognized as an effective cancer prevention strategy. In order to efficiently detect these lesions, systemic application of screening tests (surveillance) is needed. However, most of the currently used non-invasive screening tests for GI malignancies (for example, serum markers such as alpha-fetoprotein for hepatocellular carcinoma, and fecal occult blood test, for colon cancer) are only modestly effective necessitating the use of highly invasive endoscopy-based procedures, such as esophagogastroduodenoscopy and colonoscopy for screening purposes. Even for hepatocellular carcinoma where non-invasive imaging (ultrasonography) has become a standard screening tool, the need for repeated liver biopsies of suspicious liver nodules for histopathological confirmation can't be avoided. The invasive nature and high-cost associated with these screening tools hinders implementation of GI cancer screening programs. Moreover, only a small fraction of general population is truly predisposed to developing GI malignancies, and indeed needs surveillance. To spare the average-risk individuals from superfluous invasive procedures and achieve an economically viable model of cancer prevention, it's important to identify cohorts in general population that are at substantially high risk of developing GI malignancies (risk-stratification), and select suitable screening tests for surveillance in these cohorts. We herein provide a brief overview of such high-risk cohorts for different GI malignancies, and the screening strategies that have commonly been employed for surveillance purpose in them.

Key words: Gastrointestinal malignancies; Surveillance; Screening; Biomarkers; Cancer prevention

Peer reviewer: Dr. Ki Baik Hahm, Professor, Department of Gastroenterology, Gachon Graduate School of Medicine, Lee Gil Ya Cancer and Diabetes Institute, 7-45 Songdo-dong, Yeonsu-gu, Incheon 406-840, South Korea

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INTRODUCTION

Malignancies originating in the gastrointestinal (GI) tract are responsible for about one third of global cancer burden[1]. In the United States, of estimated 1 529 560 new cancer cases and 569 490 cancer deaths in 2010, approximately 274 330 new cases (approximately 18% of total) and 139 580 deaths (approximately 25% of total) could be attributed to GI malignancies[2]. One of the reasons behind relatively high mortality rate for GI cancers is their visceral location, which requires highly invasive endoscopy to directly visualize early-stage lesions, leading to diagnoses at advanced incurable stages in most cases.

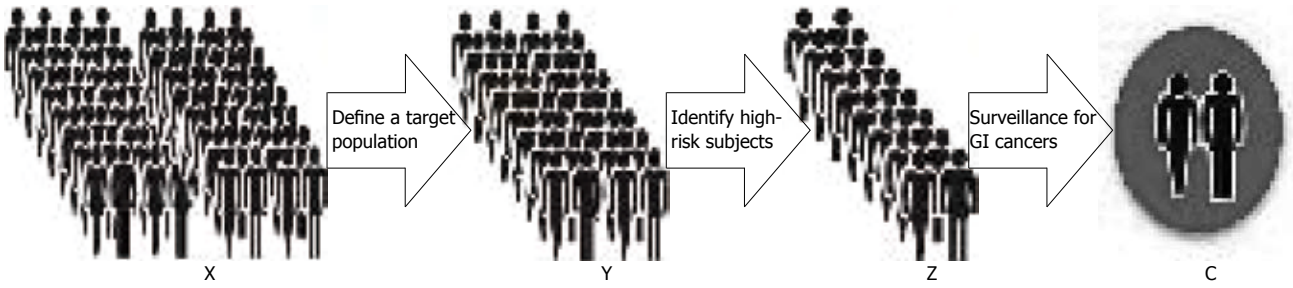

Figure 1 Risk stratification is a step-wise approach towards identifying high-risk individuals where surveillance for gastrointestinal cancers is truly needed in order to detect any neoplastic growth at early stages. The initial step is to define a target population (Y) in the general population (X) where screening strategies would be applied. Based upon findings of the screening tests in target population, subjects deemed to be at high-risk for developing a gastrointestinal (GI) malignancy (Z) would need repeated screening tests at suitable intervals (surveillance), and this would result in detection of potentially curable early stage cancerous lesions in certain individuals (C).

Secondary prevention through systemic application of screening tests (surveillance) to detect cancer-precursor/premalignant lesions is regarded an effective prevention strategy, and arguably also promotes positive life-style changes[3]. However, only a small proportion of total population is indeed at a credible risk of developing GI malignancy in future and needs surveillance. Identifying these high-risk cohorts (risk-stratification) is therefore crucial for the success of any surveillance program[4] (Figure 1). Such an approach is not only mandatory from an economic perspective but also spares the general population of repeated invasive screening tests to detect cancer precursor lesions. Screening tests detect the end-point of increased genetic/environmental predisposition (that is precursor lesions), and therefore constitute the most important arm of any risk-stratification strategy.

CANCER BIOMARKERS, SURROGATE MARKERS, LEAD-TIME BIAS AND OVERDIAGNOSIS BIAS

Biomarker is a variable that directly relates to cancer progression and/or final biological outcome (such as death), and is measured by a screening test[5]. The ease of procuring the material for biomarker analysis is an important question for visceral organs such as those in GI tract. Intraepithelial neoplasia or dysplasia remains the most reliable marker of impending malignancy, but is associated with a number of inherent limitations, including the need for invasive procedures to obtain tissue and inter-pathologist variability in interpretation of histopathological features[6]. Moreover, although endoscopic techniques have evolved over the years[7], their regular use for risk-stratification in average risk populations is economically unviable at this point. Surrogate markers, which variably correlate with cancer progression, can usually be measured through non-invasive means such as in serum or stool and therefore represent attractive tools for screening, but have limitations such as poor sensitivity and/or specificity[8]. It should be noted that surveillance programs are recommended only if an effective treatment is known to exist should any precursor/premalignant lesion be detected upon screening. This is important in order to avoid the possibility of lead-time bias (early diagnosis of cancerous lesions leading to increased number of years patient survives without actual shift in age at death) and overdiagnosis bias (diagnosis of cancerous lesions that won't have progressed, meaning they won't have caused death in first place)[9].

ESOPHAGEAL CANCER

In the United States, more than four fifth of patients diagnosed with esophageal cancer in 2010 are estimated to have a cancer death[2]. Esophageal adenocarcinoma (EA) and squamous cell carcinoma (ESCC) together account for more than 95% of esophageal cancer cases: ESCC being responsible for the bulk of cases worldwide and EA being more common in western countries, particularly among Caucasian males[10]. Due to such stark differences in the epidemiology of ESCC and EA, there is greater emphasis on risk-stratification for ESCC in developing countries, as compared to EA and its precursor lesion, Barrett's esophagus (BE), in developed countries. However, with increasing westernization of developing countries, the incidence of EA is surely on the rise in these countries too.

EA

Nearly all the cases of EA evolve through BE→Dysplasia→EA sequence[11]; and therefore, BE subjects represent a high-risk cohort where surveillance could be considered depending upon mucosal changes detected on endoscopic biopsy (reviewed in detail in reference[12]). The challenging part, however, is to identify individuals who could have BE in first place. Long-standing gastroesophageal reflux disease (GERD) patients are clearly at-risk for developing BE, but a significant proportion of BE cases can exist and progress to EA even without GERD symptoms. Furthermore, high prevalence of GERD symptoms in general populations (> 20% of adult population[13]) and extremely low rate of progression from GERD to BE to EA (approximately 0.5% and 1.0% per year respectively[14,15]) means that endoscopic screening for BE in all GERD patients would not be cost-effective[16]. However, certain subsets of GERD patients can clearly benefit from screening for BE on

an individual basis; for example, first-degree relatives of BE patients are more likely to harbor BE in presence of GERD symptoms as compared to other GERD patients with no such family history[17,18]. This increased risk could be related to genetic polymorphism of cyclin D1 and glutathione S-transferase genes which have been implicated in development of BE[19-21]. However, it's important to emphasize that there is no evidence of clear benefit of screening in asymptomatic persons (no GERD symptoms) with a positive family history of BE/EA. Overall, screening recommendations for BE remain controversial, and > 95% of EA cases are still diagnosed in patients without any prior diagnosis of BE[22]. The risk of EA (as well as ESCC) is also increased in hereditary conditions such as Peutz-Jeghers syndrome (PJS), but endoscopic surveillance in such cases is recommended for the whole upper GI tract and is not esophagus-specific.

A recent single cohort study demonstrated promising results of non-endoscopic screening for BE using an ingestible esophageal sampling device (Cytosponge) coupled with immunocytochemistry for trefoil factor 3[23]. However, non-endoscopic screening is still tested only on a limited scale, and serum markers have not been shown to be effective for screening for BE/EA as yet. Therefore, standard endoscopy with biopsy remains the "gold" standard for detecting BE. However, apart from the need of invasive endoscopy, low positive predictive value (about 34%) associated with index endoscopy[24] and need for multiple biopsies (at least 8 biopsies[25]) to diagnose metaplasia needed to define BE have been major drawbacks of endoscopy based screening. Recent advancements in endoscopic GI mucosa imaging (reviewed in reference[26]) have largely improved lesion detection capabilities, and enabled targeted biopsy of the dysplastic areas. Current recommendations for the need and frequency for EA surveillance in BE patients are largely based upon the degree of dysplasia in the BE mucosa (reviewed in detail by Badreddine *et al*[12]). In summary, after screening endoscopy in suspected BE patients (such as GERD patients over 50), detection of no dysplasia leads to repeat confirmatory endoscopy after 6-12 mo followed by endoscopic surveillance every 3 years; detection of low grade dysplasia leads to repeat confirmatory endoscopy in 6 mo followed by yearly endoscopic surveillance; and detection of high grade dysplasia needs confirmation by two expert pathologists and either 3 monthly surveillance combined with multiple biopsies spaced at every 1 cm *vs* endoscopic ablation *vs* esophageal resection.

ESCC

Currently, surveillance for ESCC is mandated only in two conditions-Tylosis palmaris (an obscure skin condition often associated with internal malignancies) and Lye ingestion[27]. However, co-existence of multiple ESCC risk factors could prompt surveillance in certain circumstances. For example, alcohol, smoking, flushing response to alcohol, Asian ethnicity, inactivating aldehyde dehydrogenase 2 allele polymorphism and personal history of any other malignancy of aerodigestive tract (UADT-which includes oral cavity, larynx, pharynx and esophagus) are all independent risk factors for ESCC[28-31]; and although none of them warrants surveillance for ESCC on its own, it would be worthwhile to consider screening for precursor lesions in persons with multiple risk factors, especially in case of Asian ethnicity, on an individualized basis. Some other conditions such as achalasia and Plummer-Vinson syndrome that are known to increase the risk of ESCC warrant endoscopic interventions for symptomatic treatment (such as for dysphagia) but not for screening[32]. Most of the reports on the impact of screening on ESCC incidence and mortality, and associated cost-effectiveness analyses have come from geographically high-risk countries. In two such studies conducted in China, investigators concluded that screening general population with exfoliative balloon cytology (EBC) was an effective tool for risk stratification and could have favorable impact on ESCC incidence and mortality[33,34]. However, United States-based Veterans' Affairs (VA) studies conducted in relatively high-risk population due to personal history and (or) symptoms produced conflicting results; and it was concluded that because of the low prevalence of ESCC in the United States and the difficulty of diagnosing malignancy in the setting of active esophagitis, EBC was probably not a cost-effective screening strategy in Western world[35]. Therefore, internationally, endoscopy aided biopsy therefore remains the standard test for ESCC screening and surveillance currently, albeit only in a limited cohort of subjects at high-risk for ESCC.

GASTRIC CANCER

Gastric cancer (GC) is the second leading cause of cancer deaths worldwide, and remains a major public health burden in Asia-pacific countries such as China, Japan and Korea where the age-standardized incidence rate for GC is > 20 per 100 000 subjects (defining criterion for high-risk areas)[1]. Gastric adenocarcinoma is the most common gastric malignancy (> 90% cases), with two subtypes: Intestinal (more common form and prevalent in high-risk areas) and diffuse type. Due to stark geographical differences in the prevalence of gastric cancer worldwide, the strategies and significance attached with screening for this cancer are highly variable.

The individuals migrating from high-risk areas remain at-risk even in low-risk countries such as the United States; however, their offspring tend to have risk levels comparable to that of the local population[36]. Geographical origin and location is therefore an extremely important consideration for any surveillance strategy against GC. Universal screening for GC has been considered only in certain high-risk countries such as Japan, South Korea and Matsu Island in Taiwan (China)[37]. On the other hand in average/low-risk countries, screening is recommended only in the presence of a well-characterized familial predisposition to GC (responsible for 1%-3% of GC cases), such as in case of hereditary

diffuse gastric cancer (HDGC) syndrome, familial adenomatous polyposis (FAP), PJS and Lynch syndrome. HDGC is the most common inherited form arising due to germline mutation in *E-cadherin* gene (*CDH1*) with the carrier of the mutations having more than 80% lifetime risk of developing GC[38]. However, surveillance or genetic testing is not considered for poorly-characterized familial cases (responsible for 8%-10% of GC cases) which are believed to be associated with more common but less penetrant defects such as polymorphism in pro-inflammatory interleukin-1 (*IL-1*) gene clusters and toll-like receptors 4 (TLR 4) + 896A > G[37,39,40]. Additionally, TLR 4 + 896A > G polymorphisms in TLR 4, a pattern recognition receptor that activates pro-inflammatory signaling pathways in response to microbes, has been associated with presence of GC and its precursors which indicates the relevance of TLR 4 polymorphism during gastric carcinogenesis[41]. A meta analysis of the role of IL-1b and IL-1 receptor antagonist gene polymorphisms in gastric cancer risk showed an association in Caucasians, but not in Asians[40]. Similarly, a metaanalysis by Huang *et al*[42] concluded that *cag A* seropositivity significantly increased the risk for gastric cancer and could be used for identifying populations at risk for GC. However, despite high prevalence of *cagA* in Asia-Pacific regions, the currently known *cagA* genotypes in Asia are not associated with increased GC risk[43]. Other high-risk subgroups considered for screening on a case-to-case basis are elderly patients with atrophic gastritis or pernicious anemia, patients with partial gastrectomy, patients with the diagnosis of sporadic adenomas, and immigrant ethnic populations from GC high-risk countries.

Over ninety percent of GC cases are sporadic, and most are linked to *Helicobacter pylori* (*H. pylori*) infection[44,45]. A meta-analysis of six major studies on *H. pylori* eradication demonstrated that *H. pylori* "screen-and-treat" strategy reduced the incidence of GC[46]. Based on this, the Asia-Pacific Gastric Cancer Consensus Conference in 2008 concluded that it might perhaps be the right time for a population-based screening and treatment of *H. pylori* infection (by using locally approved screening tests for *H. pylori,* such as serum or stool antibody/antigen detection), particularly in high-risk areas as a part of GC prevention program[37]. Interestingly, Ford *et al*[47] have proposed that even in western countries where better sanitation, low-salt intake and effective treatment of *H. pylori* infection has led to gradual decline in GC incidence over decades, a "screen and treat" strategy for *H. pylori* could reduce the dyspepsia-related health care costs over a longer (10 years or more) follow-up duration. However, prospective trials on a global scale are needed to validate such observations; and currently, no screening for *H. pylori* is recommended for asymptomatic individuals in geographically low/average-risk areas. Gastric cancer phenotype initiated due to *H. pylori* is characterized structurally by a corpus predominant gastritis, multifocal gastric atrophy, intestinal metaplasia, and physiologically by high gastrin, low acid secretion, low pepsinogen I and pepsinogen I / Ⅱ ratio, and hypo-and achlorhydria[48-50]. All these findings have been used to design screening tests for GC, such as serum pepsinogen I levels and pepsinogen I / Ⅱ ratios that have been investigated in high-risk areas, but have limited usefulness on a global scale[51]. Currently, endoscopy aided with advanced imaging techniques and biopsy (at least 5) to look for precursor lesions remains the main tools for screening and surveillance for GC.

PANCREATIC CANCER

Pancreatic cancer (PC) is the most aggressive GI malignancy that silently progresses to untreatable metastatic disease in most cases, and is generally fatal within six mo of diagnosis[2]. However, interestingly, a study from Japan has demonstrated that resection of all pancreatic lesions < 1 cm in size can achieve about 100% cure rates[52], suggesting that a thorough surveillance program for PC could potentially be useful. However, this approach leads to unnecessary high-risk surgical resection of many benign lesions that won't have progressed to malignancy in first place. Therefore, identifying high-risk cohorts where such pancreatic lesions are more likely to be malignant is definitely a better-refined strategy for prevention. Such high-risk cohorts for PC (defined as having > 10-fold increased risk of PC as compared to the general population) include familial and/or syndromic cases (3%-16% of total cases) where screening is routinely recommended (Figure 1)[53-56]. However, screening strategy for individuals at 5- to 10-fold increased risk of pancreatic cancer (e.g., those with just one or two affected first-degree relatives) is unclear. Clearly, in such cases, most centers take individualized approaches depending upon the cost and other considerations. Future studies are needed to establish the risk threshold at which screening is likely to be most cost-effective.

Notably, conditions such as chronic pancreatitis, diabetes mellitus and smoking history have strong associations with PC, but none of them increases the risk to an extent that could warrant screening.

Screening for PC faces a unique challenge in terms of incidental radiological findings in the pancreas due to rampant use of computerised tomography (CT) scan in patient-care. Because many of these lesions are non-lethal, it's important to establish their malignancy potential in order to guide their management and avoid over-enthusiastic and sometimes unwarranted surgeries that could ensue otherwise[57]. The most common of these lesions are intraductal papillary mucinous neoplasms (IPMNs). IPMNs which involve the main duct have a 70% risk of containing a malignancy at the time of diagnosis and need to be resected[58], while those involving the branch ducts have 25% risk of containing malignancy and 15% risk of malignant transformation during follow-up, and they can be safely observed with continued surveillance[57,58]. Certain other features however necessitate immediate resection, such as diameter ⩾ 3 cm, a mural nodule appearance, main pancreatic duct dilation ⩾ 6 mm, progressively changing lesion characteristics,

or presence of symptoms[57,58]. In general, the approach is usually much more aggressive if such lesions are present in high-risk individuals[55].

Another variety of PC precursor lesions, although not detectable by routine imaging tests in a clinical setting, are pancreatic intra-epithelial neoplasia (PanIN)[59]. PanIN is a histological diagnosis where pro-cancerous genetic and epigenetic aberrations have been noticed. PanIN-3 lesions are essentially treated as PC and resected whereas PanIN-1 lesions have very small risk of malignancy, and can be safely followed-up[60]. The management of PanIN-2 lesions is controversial and recommendations depend upon co-existing conditions and cost-considerations.

Currently, endoscopic ultrasonography (EUS) is most efficient screening test for PC; it accurately identifies pancreatic cysts and IPMNs, and has the advantage of detecting structural changes somehow predictive of PanIN lesions[61]. Other screening modalities like CT and endoscopic retrograde cholangiopancreatography have fallen out of favor mainly due to low sensitivity and radiation exposure and high incidence of pancreatitis respectively. However, magnetic resonance imaging/magnetic resonance cholangiopancreatography (MRCP), especially secretin-enhanced MRCP, is still an acceptable alternative to EUS[62]. In the absence of any clear guidelines for the frequency and starting age for screening for pancreatic cancer, recommendations are highly institutionalized based upon factors such K-ras mutations, family history *etc*.[63-66].

HEPATOCELLULAR CARCINOMA

Hepatocellular carcinoma (HCC) is the most common primary liver malignancy, and is increasing in incidence in the United States[2]. In general, 5-year survival is less than 10% if diagnosed in symptomatic patients, but HCC diagnosis prior to appearance of symptoms offers the curative opportunities through resection of tumor[67]. Although extremely rare in asymptomatic populations, HCC is a dreaded long-term complication of most chronic liver diseases (CLD); and therefore, CLD patients constitute the obvious target population for risk-stratification. However, the level of HCC risk varies in different CLDs; and due to the generally protracted course of CLDs and high cost of repeated screening, cost-considerations are very important in formulating surveillance strategies in these patients. Among CLD patients, diagnosis of cirrhosis is a strong predictor of the risk of progression to HCC, and advent of cirrhosis often marks the starting point for surveillance recommendations in these patients. Chronic viral hepatitis (hepatitis B and C) is the most common etiology behind HCC. Hepatitis B and C carriers have an HCC incidence rate of 0.2%-0.6% per year, and 3%-11% per year respectively[68,69]. Hepatitis B can particularly be deceptive because HCC can occur even in non-cirrhotic hepatitis B virus (HBV) carriers, and the risk seems to be variable. Asian ethnicity, viral replication status, and seropositivity for hepatitis B surface antigen and anti-HBe antigen differentially influence the risk of development of HCC in HBV carriers, and therefore have an important impact on surveillance program (reviewed in references[70-73]). On the other hand, the risk of HCC in long-term hepatitis C carriers is independent of factors such as ethnicity and viral replication status[71,74], and largely depends upon the extent and severity of cirrhosis. Based upon this, the first European association for study of the liver conference on HCC recommended screening for HCC in chronic hepatitis C patients with at least stage 3 fibrosis (METAVIR)[75]. Another puzzling question is whether surveillance for HCC should continue in successfully treated chronic viral hepatitis patients? Evidence has been conflicting for hepatitis B carriers with mostly Western and some Asian studies suggesting a significant reduction in HCC risk after successful treatment[76-78], and a non-randomized, but match controlled Asian study following a large cohort for longer periods suggesting continued risk of HCC post treatment[79]. Thus, given the higher risk associated with Asian ethnicity, it seems prudent to continue HCC surveillance in Asian hepatitis B carriers with cirrhosis even after successful seroconversion. However, the same can't be said about non-cirrhotic western populations. In contrast, continued surveillance for HCC is recommended for hepatitis C infected population because even after successful treatment (i.e., sustained virological response), the risk of HCC in cirrhotic hepatitis C carriers remains sufficiently high to warrant surveillance[74]. Additionally, co-existing risk factors like old age, viral genotype, viral replication status, aflatoxin exposure, co-infection and other CLDs, diabetes and human immunodeficiency virus also need to be taken into account in deciding the surveillance protocol for HCC in viral hepatitis carriers. Additionally, there are some non-viral cirrhotic conditions as well where surveillance could be considered (Table 1).

For screening purposes, ultrasonography (USG) and serum alpha-fetoprotein levels are often used. Serial USG (at 6-12 mo interval[80,81]) has by far been superior to any other screening test for HCC (65%-80% sensitivity and 90% specificity), and can detect nodules of 1 cm size, which are essentially curable[82]. However, USG needs to be aided with biopsies to differentiate between benign cirrhotic and dysplastic/malignant nodules. Conversely, evidence suggests that serum alpha-fetoprotein measurement has no role in HCC screening (although it can still have some utility in HCC diagnosis and follow-up), and should be no longer used for screening purposes. Other serological markers such as alpha flucosidase, glypican-3 and desgamma carboxyprothombin have already been discredited[83].

COLORECTAL CANCER

Colorectal cancer (CRC) is the third most common cancer in both men and women[2]. Over the years, its incidence has constantly been decreasing, largely due to colonoscopic screening. As less than one third of CRC cases are as-

Table 1 At-risk cohorts for considering surveillance for gastrointestinal malignancies

Esophageal cancer
Barrett's esophagus
Tylosis palmaris
Lye ingestion
Head and Neck tumors patients with flushing response/inactive ALDH1 allele
Gastric cancer
Hereditary diffuse gastric cancer
Lynch syndrome
Peutz-Jeghers syndrome
Juvenile polyposis syndrome
Li-Fraumeni syndrome
Atrophic gastritis/pernicious anemia
Post-partial gastrectomy
Sporadic adenoma
18-60 yr old Inhabitants of high-risk areas
Pancreatic cancer
Hereditary pancreatitis
Peutz-Jeghers syndrome
Familial pancreatic cancer kindred (≥ 1 first-degree relative and ≥ 3 first, second or third degree relative with pancreatic cancer)
Familial atypical multiple mole melanoma
Familial breast-ovarian cancer
Hereditary nonpolyposis colorectal cancer (Lynch syndrome)
Familial adenomatous polyposis (FAP)
Cystic fibrosis
Fanconi anemia
Ataxia telangiectasia
Incidentally discovered IPMN/PanIN lesions
Hepatocellular carcinoma
Hepatitis B carriers (Asians and Africans)
Hepatitis B cirrhosis
Family history of HCC (mainly Asians and Africans)
Treated hepatitis B cirrhosis (Asians)
Hepatitis C cirrhosis
Treated hepatitis C cirrhosis
Alcoholic cirrhosis
Genetic hemochromatosis
Alfa1-antitrypsin deficiency
Primary biliary cirrhosis
Colorectal cancer
Familial adenomatous polyposis
Attenuated FAP (AFAP)
Hereditary nonpolyposis colorectal cancer (Lynch syndrome)
Peutz-Jeghers syndrome
Juvenile polyposis syndrome
MUTYH-associated polyposis
Hyperplastic polyposis
Patients with long-standing IBD
Acromegaly patients
Positive findings on index colonoscopy (at 50 yr) such as three or more tubular adenomas, tubular adenoma > 10 mm, adenoma with villous histology, adenoma with high-grade dysplasia, after surgical removal of invasive cancer, incomplete removal of neoplastic lesion

IPMN: Intraductal papillary mucinous neoplasms; PanIN: Pancreatic intraepithelial neoplasia; HCC: Hepatocellular carcinoma; IBD: Inflammatory bowel disease; ALPH1: Aldehyclen dehydrogenase 1; MUTYH: Human MutY homolog.

sociated with any kind of familial predisposition and even lesser proportions (< 5%) belong to well-defined inherited syndromes (such as Lynch syndrome, FAP *etc*.[84]), screening colonoscopy at age 50 (also called index colonoscopy) is the main tool of CRC risk-stratification in general[85]. Presence of adenomas on index colonoscopy is a strong predictor of the risk of development of additional adenomas (30%-50% detection rate at follow up after clearance colonoscopy[86]) and CRC in future. However, most adenomas don't progress to cancer (the life-time cumulative incidence of CRC is 5.5%, and prevalence of colonic adenomas at age 60 is 30%-40%[87]), and therefore their size, numbers, morphology and histopathological characteristics are used to assess the relative risk of progression to cancer and the need of follow-up surveillance/treatment strategies.

In individuals with familial predisposition, the average life-time risk of CRC varies from 100% in FAP to 20% in persons with first and/or second degree relatives with CRC[88], due to difference in penetrance of the inherited genetic defects. It is estimated that only 5% of CRC cases are associated with highly penetrant inherited mutations with well-characterized clinical presentation such as FAP, Lynch syndrome *etc*. whereas rest belong to less penetrant but far more common genetic defects such a polymorphisms in CYP450 family, glutathione-S-transferase family, insulin-like growth factor binding protein-3, ornithine decarboxylase-1 and transforming growth factor-beta receptor 1 genes[84]. However, currently the genetic testing is recommended only if a well-characterized familial syndrome (e.g., Lynch syndrome, FAP) is suspected, because the epidemiological data of the relative-risk of CRC associated with gene polymorphisms is still limited.

Two other well-established high-risk cohorts where surveillance for CRC is recommended are patients with long standing inflammatory bowel disease (IBD) and acromegaly patients. Recent reports suggest that both ulcerative colitis and Crohns' disease patients are at comparable cumulative risk of CRC if the extent and duration of the disease are the same (in case of ulcerative colitis, risk of CRC stands at 1.6% at 10 years, 8.3% at 20 years and 18.4% at 30 years[89,90]). For IBD patients with colonic disease, screening is recommended after 10 years of disease history, and involves endoscopic evaluation of inflammatory changes in the mucosa combined with multiple biopsies to detect dysplasia[91]. Acromegaly patients, on the other hand, seem to have increased incidence as well as propensity for malignant transformation of adenomas, especially right-sided ones, as compared to the general population (odds ratio: 2.4 for adenoma, 7.4 for CRC[92]). This could possibly be attributed to the presence of elevated serum insulin growth factor-1 level (seen in > 90% of acromegaly patients), which has been shown to increase the risk of CRC in non-acromegalic population[93]. Additionally, colonoscopic screening starting at age 40 is recommended to detect precursor lesions in such patients.

From a screening test perspective, non-invasive screening tests such as stool tests (occult blood and DNA tests), imaging (CT, Barium enema) and sigmoidoscopy are only occasionally used, and a full-length colonoscopy despite its several limitations remains the most effective and preferred screening test for CRC (reviewed in details in reference[85]). Current guidelines recommend

screening colonoscopy in average risk individuals at age 50, a significant deviation from earlier practice of colonoscopic screening only in high-risk individuals[94]. The rationale for colonoscopic surveillance has always been based on the high detection rate of colorectal adenomas at follow up (30%-50%) after a complete clearance colonoscopy[86]. However, the main object of colonoscopic surveillance is the prevention of subsequent colorectal cancer rather than the detection and removal of adenomas, most of which will not become malignant. Adenomas with advanced pathology (> 1 cm, with villous elements or severe dysplasia) have a much higher malignant potential, and the main objective of screening is to ensure that such lesions are detected before they become invasive. Therefore, individuals with 1-2 small polyps < 1 cm size and no villous morphology at index colonoscopy are considered low-risk and need no modification in surveillance protocol. However, certain findings on index colonoscopy (as mentioned in the Table 1) indicate high-risk of CRC and necessitate enhanced surveillance.

FUTURE PERSPECTIVE

Currently, surveillance for GI malignancies is challenging because of the general lack of inexpensive screening tests and potent biomarkers that could efficiently identify high-risk cohorts. Recently, there has been a surge in interest in using a panel of biomarkers (gene expression signatures) for screening purposes, but their impact on cancer mortality remains to be tested in large-scale studies[95,96]. Another new class of biomarkers under investigation these days are miRNAs, a type of non-coding RNAs that are endogenous silencers of target genes[97,98]. Unfortunately, many biomarkers/screening tests with initial promise indeed fail to meet the Early Detection Research Network-outlined criteria for their validation[99], and therefore are not used clinically. From a futuristic perspective, we are standing at the crossroads of a major change in our approach towards cancer prevention. With completion of the human genome project, rapid advances in deep sequencing technology and better understanding of the genetic landscape of different tumors (including GI cancers), it is being expected that it would be possible to assess the cumulative predisposition to different cancers in every individual in a cost-effective manner, leading to a highly individualized treatment and preventive care (Personalized Medicine) in coming years[100].

SEARCH STRATEGY AND SELECTION CRITERIA

References for this review were identified through searches of PubMed with the following search terms: "Gastrointestinal malignancies", "risk stratification", "gastrointestinal cancer screening/surveillance", "cancer biomarker", and "cancer prevention" published before December 2011. Articles were also identified through searches of the authors' own files. The final reference list was generated on the basis of relevance to the broad scope of this review; and only articles published in English were reviewed, and considered for inclusion in the reference list based upon the further reading opportunity they offered.

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REVIEW

Management of chronic hepatitis B in pregnancy

Guo-Rong Han, Chuan-Lu Xu, Wei Zhao, Yong-Feng Yang

Guo-Rong Han, Chuan-Lu Xu, Department of Gynecology and Obstetrics, The Second Affiliated Hospital of the Southeast University, Nanjing 210003, Jiangsu Province, China
Wei Zhao, Yong-Feng Yang, Department of Infectious Diseases, The Second Affiliated Hospital of the Southeast University, Nanjing 210003, Jiangsu Province, China
Author contributions: Han GR designed the study and wrote the manuscript with input from the others; Xu CL provided writing assistance; Han GR, Xu CL, Zhao W and Yang YF participated in data collection; Han GR and Xu CL performed the data analysis; and Han GR interpreted the data, wrote the draft and critically revised the manuscript.
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Correspondence to: Guo-Rong Han, MD, Director of the Department of Gynecology and Obstetrics, The Second Affiliated Hospital of the Southeast University, No.1 Zhongfu Road, Nanjing 210003, Jiangsu Province, China. hanguorong2011@sina.com
Telephone: +86-25-83626372 Fax: +86-25-83626060
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Abstract

Pregnancy associated with chronic hepatitis B (CHB) is a common and important problem with unique challenges. Pregnant women infected with CHB are different from the general population, and their special problems need to be considered: such as the effect of hepatitis B virus (HBV) infection on the mother and fetus, the effect of pregnancy on replication of the HBV, whether mothers should take HBV antiviral therapy during pregnancy, the effect of these treatments on the mother and fetus, how to carry out immunization of neonates, whether it can induce hepatitis activity after delivery and other serious issues. At present, there are about 350 million individuals with HBV infection worldwide, of which 50% were infected during the perinatal or neonatal period, especially in HBV-endemic countries. Currently, the rate of HBV infection in the child-bearing age group is still at a high level, and the infection rate is as high as 8.16%. Effective prevention of mother-to-child transmission is an important means of reducing the global burden of chronic HBV infection. Even after adopting the combined immunization measures, there are still 5%-10% of babies born with HBV infection in hepatitis B e antigen positive pregnant women. As HBV perinatal transmission is the main cause of chronic HBV infection, we must consider how to prevent this transmission to reduce the burden of HBV infection. In this population of chronic HBV infected women of childbearing age, specific detection, intervention and follow-up measures are particularly worthy of attention and discussion.

Key words: Chronic hepatitis B; Hepatitis B virus; Mother-to-child transmission; Perinatal transmission; Pregnancy; Vertical transmission; Antiviral therapy

Peer reviewers: Epameinondas Tsianos, Professor of Internal Medicine, Department of Medicine, University of Ionnina, 45110 Ioannina, Greece; Dr. Richard A Rippe, National Institutes of Health, 5635 Fishers Lane, Rockville, MD 20852, United States; Stephan Menne, Research Associate Professor, Microbiology and Immunology, Georgetown University, Georgetown University Medical Center, 3900 Reservoir Rd., Washington, WA 20057, United States

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INTRODUCTION

Chronic hepatitis B (CHB) in pregnancy is an important and pervasive issue with unique challenges. However, for pregnant women with chronic hepatitis B virus (HBV) infection, unlike the general population, many special

problems need to be considered, such as the influence of HBV infection on the mother and fetus, influence of pregnancy on HBV replication, effects of antiviral treatment on maternal and neonatal outcomes, immunization of newborns and the possible flare of hepatitis after delivery. Approximately 350 million people are infected with HBV worldwide and 50% of them have acquired their infection in the perinatal or neonatal period, especially in countries where HBV has a high prevalence[1,2]. In these countries, women of childbearing age have a higher hepatitis B e antigen (HBeAg)-positive rate and a higher probability of mother-to-infant transmission, the younger they are when infected with HBV, the higher the risk of developing CHB[1,3,4]. The rate of HBV infection among women of childbearing age is still at a high level (7.18%) in China[5-7], which leads to an increased risk of HBV vertical transmission and damage due to CHB in the mother and fetus in pregnancy. Therefore, the effective prevention of vertical transmission of HBV is an important approach in reducing the global burden of CHB. Specific hepatitis B immunoglobulin (HBIG) is available for passive protection and is normally used in combination with hepatitis B vaccine to confer immediate cover (passive immunity) and long-lasting protection (active immunity) in newborns, which is administered as an effective prophylactic measure to prevent mother-to-infant transmission of HBV, however, 5%-10% infants of HBeAg-positive mothers are still infected with HBV[8-10].

PREGNANCY AND CHRONIC HBV INFECTION

Overall, no severe effects due to CHB are found in pregnancy. Studies have shown that chronic HBV infection is associated with gestational diabetes mellitus, antepartum hemorrhage, threatened premature labor and lower Apgar score. Mothers with seriously abnormal liver function complications are prone to postpartum hemorrhage, puerperal infection, low body weight infants, fetal distress, premature birth, fetal death and neonatal asphyxia[11-21]. A series of physiological changes occur during pregnancy, including vigorous metabolism and increased nutrient consumption. These changes occur to promote the metabolic needs of the mother as well as the needs of the growing fetus. Abundant sex hormone produced by the mother needs to be metabolized and inactivated in the liver, and metabolism and detoxification in the fetus also depend on the mother's liver, which correlates with aggravation of pre-existing liver diseases and exacerbation of liver damage[22,23]. Alanine aminotransferase (ALT) in late pregnancy and the postpartum period shows an increasing tendency, however, HBV replication in the gestational period is not noticeably different[24-28]. Some women appear to undergo HBeAg seroconversion in the initial months after delivery if immune activation occurs, with a seroconversion rate of 12.5% to 17%, which is correlated with an obvious decrease in adrenal cortex hormone[29,30]. Although HBV infection during pregnancy can often be tolerated, severe hepatitis and hepatic failure induced by perinatal hepatic flare reactions still occur, and can have an unfavorable outcome[29].

PERINATAL MANAGEMENT OF CHB

As relatively safe assays for the diagnosis of HBV infection are available and effective treatment strategies for CHB have been developed, the screening of HBV infection in the perinatal period has become standard care, which can identify newborns that require prophylaxis with hepatitis B vaccine and HBIG as well as pregnant women who require antiviral therapy. In addition, it is beneficial to advise patients with hepatitis B about sexual and family contacts. Screening and vaccination are key factors in the successful prevention and control of HBV infection.

Women with CHB should actively plan their pregnancy and undergo baseline evaluations, such as hepatitis B surface antigen (HBsAg), HBeAg, antibody to HBeAg (anti-HBe), HBV DNA, severity of liver disease and the presence of other viral infections, are suggested before pregnancy. The ability to sustain pregnancy and the risk of vertical transmission of HBV in women with CHB should also be evaluated. All pregnant women should be screened for HBV infection at the first prenatal examination, and HBsAg-positive patients should be transferred to hospitals with experience in the management of CHB for easier monitoring of mothers in pregnancy, delivery and the postpartum period as well as newborns, and appropriate prevention of mother-to-infant transmission of HBV based on an individual's condition should be conducted.

PREVENTION AND TREATMENT OF CHRONIC HBV INFECTION IN PREGNANCY

The treatment goals for CHB in pregnancy are to achieve stabilization of liver function in mothers and prevent HBV infection in newborns. Regular monitoring of liver function and HBV DNA level should be performed in the gestational period to determine whether liver disease is progressing and antiviral therapy is needed in mothers. Sinha *et al*[11] from India made several suggestions aimed at Asian HBV carriers when planning a pregnancy: First, in patients with lower HBV DNA level at baseline (HBV DNA $< 10^6$ copies in HBeAg-positive patients and HBV DNA $< 10^5$ in HBeAg-negative patients) and no obvious fibration, antiviral therapy may be delayed, but monitoring should be performed during pregnancy. In patients with HBV DNA $> 10^7$ copies/mL repeated in the late trimester of pregnancy, or prior delivery history of a HBV-positive infant and HBV DNA $> 10^6$ copies/mL, antiviral therapy should be administered; Second, in patients with higher HBV DNA level at baseline and obvious fibration, but without liver cirrhosis, antiviral

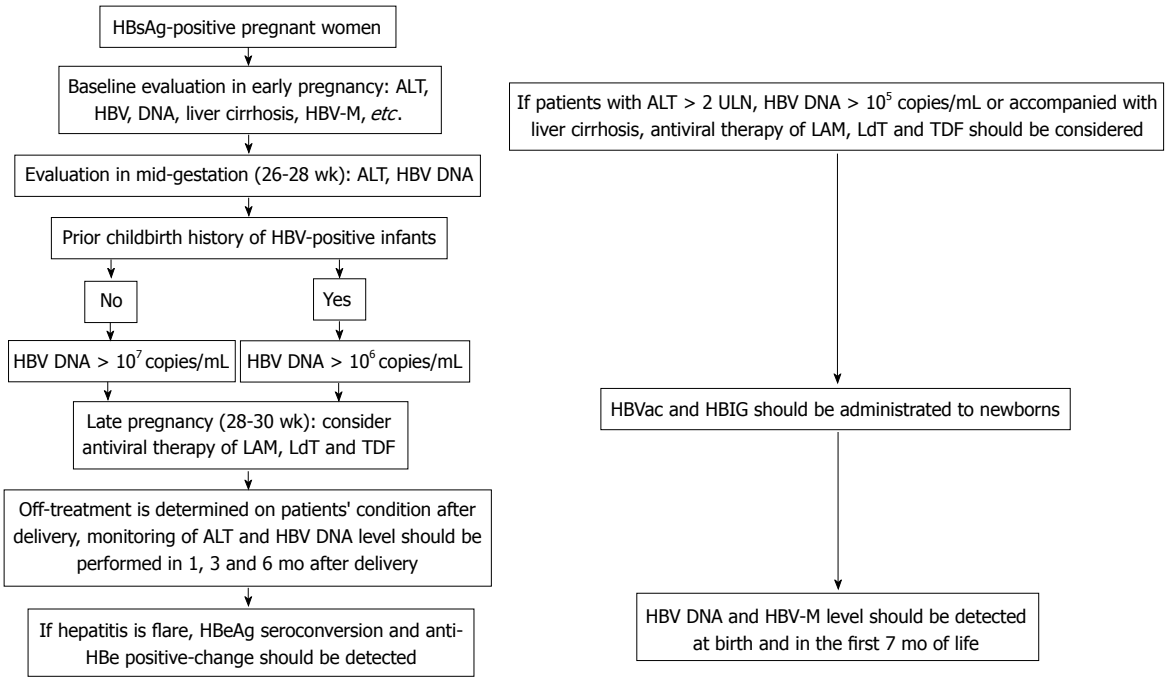

Figure 1 Management of chronic hepatitis B in pregnancy. HBsAg: Hepatitis B surface antigen; ALT: Alanine aminotransferase; HBV: Hepatitis B virus; LAM: Lamivudine; LdT: Telbivudine; TDF: Tenofovir; HBeAg: Hepatitis B e antigen; Anti-HBe: Antibody to HBeAg; ULN: Upper limits of normal; HBVac: Hepatitis B vaccine; HBIG: Hepatitis B immunoglobulin.

therapy is suggested. If a response is sustained after off-treatment, pregnancy is feasible, monitoring should be performed, and management is the same as that outlined in the first scenario; If a response is not sustained after drug withdrawal, management is the same as the third scenario; Third, If patients have liver cirrhosis before pregnancy, antiviral therapy [lamivudine (LAM), tenofovir (TDF) or telbivudine (LdT)] is suggested first, and one of these drugs should be continued during pregnancy, and monitoring should also be conducted.

To effectively prevent HBV infection in infants, delivery methods are also believed to be potential risk factors for mother-to-infant transmission[31,32], however, there is no clear evidence to show that delivery methods are correlated with reduced vertical transmission of HBV[33-35]. Immediate vaccination with HBVac in combination with HBIG in infants born to CHB mothers can effectively prevent infection in the labor and postnatal period, but has no effect on intrauterine infection[36-38], which is the primary cause leading to failure of vaccination. As both HBV intrauterine and perinatal transmission is significantly correlated with HBV DNA level in pregnant women[39-45], currently most attention is focused on oral antiviral drugs in late pregnancy, which reduce HBV intrauterine transmission by decreasing HBV DNA titers in peripheral blood before delivery[46-48].

PROBLEMS CORRELATED WITH ANTIVIRAL THERAPY DURING PREGNANCY

The current difficulty in preventing mother-to-infant transmission of HBV is the prevention of intrauterine transmission. High HBV DNA level is the most important independent risk factor for intrauterine transmission, thus pregnant woman can take oral antiviral drugs in late pregnancy to reduce HBV DNA titers in peripheral blood before delivery and decrease HBV intrauterine transmission.

Selection of antiviral drugs

Due to inhibition of cell proliferation, interferon is contraindicated in pregnant women. For patients using interferon, pregnancy is practicable after discontinuation of interferon for 6 mo. LdT and TDF are authorized category B antiviral drugs by the Food and Drug Administration for use in pregnancy. After reviewing the increasing safety data on LAM in clinical practise[49-52], LAM was elevated to a category B antiviral drug in pregnancy by NIH, that is, the category B antiviral drugs in pregnancy include LAM, LdT and TDF[33,53].

Indication for antiviral therapy

For HBsAg-positive pregnant women by screening, baseline evaluation, such as HBV-M (HBsAg, HBeAg, anti-Hbe), HBV DNA, hepatitis activity and severity of hepatic fibrosis/liver cirrhosis, are suggested in early pregnancy[54-56]. In patients with higher HBV DNA level and hepatitis activity (ALT > 2 upper limit of normal, HBV DNA > 10^5 copies/mL) at baseline or accompanied by liver cirrhosis, antiviral therapy should be administered during early pregnancy. For patients with normal liver function, ALT and HBV DNA level should be reevaluated in mid-gestation (26-28 wk). For patients with HBV DNA > 10^7 copies/mL or prior delivery his-

tory of HBV-positive infants and HBV DNA > 10^6 copies/mL, antiviral therapy (LAM, TDF or LdT) should be given at 28-30 wk until 4 wk after delivery, then it should be determined whether the above therapy is to be continued on the basis of the patient's condition; otherwise antiviral therapy should not be given[57]. ALT and HBV DNA level should be monitored in all HBsAg-positive pregnant women at 1, 3 and 6 mo after delivery. In a hepatitis flare, HBeAg seroconversion and anti-Hbe positive-change should be detected[58]. Active-passive immunization should be performed in all newborns on schedule; HBV-M (HBsAg, HBeAg, anti-HBe) and HBV DNA level should also be detected at birth and in the first 7 mo of life in newborns[59]. In patients with liver cirrhosis before pregnancy, antiviral therapy (LAM, TDF or LdT) are suggested first, one of these drugs should be continued during pregnancy, and monitoring should also be conducted (Figure 1).

Individualized management of CHB in pregnancy

For women with an unplanned pregnancy during the course of antiviral therapy for CHB, individualized management is performed according to the actual condition of the patient. There are two options for patients: one is temporal off-treatment and whole course monitoring of HBV DNA and ALT level, in addition, antiviral therapy is based on the patient's actual condition after pregnancy, which is suitable for patients with mild hepatitis and a lower risk of recurrence and disease progression; the other option is sequential use of LAM, TDF or LdT as antiviral therapy during the whole course[11,36]. Although active-passive immunity should be given to all newborns of HBsAg-positive pregnant women, breast feeding does not increase the risks of HBV infection. However, there is not enough safety data on these drugs with regard to newborn exposure during breast feeding to assess whether patients receiving antiviral therapy should breastfeed their children [57].

Overall, HBV perinatal transmission is a major cause of chronic HBV infection. To reduce the burden of HBV infection, we must consider how to prevent HBV transmission. For women of childbearing age, HBV detection and intervention deserves special attention and investigation.

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ORIGINAL ARTICLE

Loss of fragile histidine triad and amplification of 1p36.22 and 11p15.5 in primary gastric adenocarcinomas

Yuan-Yuan Liu, Hai-Ying Chen, Man-Li Zhang, Dan Tian, Shibo Li, Ji-Yun Lee

Yuan-Yuan Liu, Man-Li Zhang, Dan Tian, Department of Internal Medicine, The First Teaching Hospital of Jilin University, Changchun 130021, Jilin Province, China
Hai-Ying Chen, Department of Infectious Diseases, The First Teaching Hospital of Jilin University, Changchun 130021, Jilin Province, China
Shibo Li, Ji-Yun Lee, Department of Pediatrics, The University of Oklahoma Health Sciences Center, Oklahoma City, OK 73104, United States
Ji-Yun Lee, Department of Pathology, College of Medicine, Korea University, Seoul 136-705, South Korea
Author contributions: Liu YY performed the data analysis; Chen HY performed the data collection; Zhang ML and Tian D collected the samples; Li S designed the study and provided financial support; Lee JY designed the study and wrote the manuscript.
Correspondence to: Ji-Yun Lee, PhD, Department of Pathology, College of Medicine, Korea University, 5-1 Anam-Dong, Seoungbuk-Gu, Seoul 136-705,
South Korea. jiyun-lee@korea.ac.kr
Telephone: +82-2-9206141 Fax: +82-2-9533130
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Abstract

AIM: To investigate the genomic copy number alterations that may harbor key driver genes in gastric tumorigenesis.

METHODS: Using high-resolution array comparative genomic hybridization (CGH), we investigated the genomic alterations of 20 advanced primary gastric adenocarcinomas (seventeen tubular and three mucinous) of Chinese patients from the Jilin province. Ten matching adjacent normal regions from the same patients were also studied.

RESULTS: The most frequent imbalances detected in these cancer samples were gains of 3q26.31-q27.2, 5p, 8q, 11p, 18p, 19q and 20q and losses of 3p, 4p, 18q and 21q. The use of high-resolution array CGH increased the resolution and sensitivity of the observed genomic changes and identified focal genetic imbalances, which included 54 gains and 16 losses that were smaller than 1 Mb in size. The most interesting focal imbalances were the intergenic loss/homozygous deletion of the fragile histidine triad gene and the amplicons 11q13, 18q11.2 and 19q12, as well as the novel amplicons 1p36.22 and 11p15.5.

CONCLUSION: These regions, especially the focal amplicons, may harbor key driver genes that will serve as biomarkers for either the diagnosis or the prognosis of gastric cancer, and therefore, a large-scale investigation is recommended.

Key words: Array comparative genomic hybridization; Amplicon; Gastric adenocarcinoma; Oncogene; Fragile histidine triad

Peer reviewers: Hitoshi Tsuda, MD, PhD, Diagnostic Pathology Section, Clinical Laboratory Division, National Cancer Center Hospital, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045, Japan; Dr. Debra F Saxe, Department of Pathology and Laboratory Medicine, Emory University, Atlanta, GA 30322, United States

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INTRODUCTION

Gastric cancer is one of the leading causes of cancer-related death worldwide. Although its incidence has gradu-

ally decreased in many Western countries, the incidence of gastric cancer still remains high in South and Central America and is highest in Eastern Asia, specifically in China, South Korea and Japan[1,2]. The most common gastric malignancy is adenocarcinoma[3], which is characterized by multiple genetic instabilities, as are other adenocarcinomas. One of these genetic instabilities is chromosomal instability, a common consequence of a chromosomal or chromosome-segment abnormality, that causes DNA copy number changes during tumor progression. These alterations may lead to a loss of function of tumor suppressor genes (inactivation) and/or a gain of function of oncogenes (activation). High-level DNA copy number changes (amplification/amplicons) in tumors are frequently restricted to certain chromosomal regions containing well-known oncogenes that are also overexpressed or activated[4,5]. Some oncogenes, such as *NMYC*, *LMYC* and *GLI*, were originally discovered because of their genomic amplification in human tumors[4]. An analysis of the composition of DNA amplifications showed that human cancer can be classified *via* DNA copy number profiling because such amplifications are non-randomly selected with respect to the biological backgrounds of cancer[6]. Therefore, the detection and discovery of unidentified or incompletely described amplicons and relevant genes located within these amplicons can lead to the identification of genes putatively involved in growth control and tumorigenesis.

Recently available whole-genome array comparative genomic hybridization (CGH), a high-throughput genomic technology, facilitates the accumulation of high-resolution data of genomic imbalances associated with disease. In this study, we identified possible candidate genes that could provide insight into the pathology of gastric adenocarcinoma through the integration of genomic copy number changes.

MATERIALS AND METHODS

Tumor samples

This study included seventeen tubular and three mucinous adenocarcinomas of advanced primary stomach cancer samples from Jilin Province in North-Eastern China (Table 1). Of the twenty samples studied, thirteen were from males and seven were from females. The mean age was 62.1 (ranging from 52 to 76) years. The stage of each tumor was classified according to the tumor node metastasis classification of the International Union Against Cancer. The histopathological grades were as follows: Grade 1 (well-differentiated/low grade adenocarcinoma), no cases; Grade 2 (moderately differentiated/intermediate-grade adenocarcinoma), five (tubular) cases; and Grade 3 (poorly differentiated/high-grade adenocarcinoma), 15 (12 tubular and three mucinous) cases. Unfortunately, we were unable to obtain information concerning postsurgical pathological stages. Tumor samples were obtained surgically in the First Hospital of Jilin University; paired adjacent normal tissue was also collected as a control for comparison with the tumor. All patients had negative histories of exposure to either chemotherapy or radiotherapy before surgery, and there were no other diagnosed cancers. An informed consent with approval of the ethics committee of the First Hospital of Jilin University was obtained from all participating patients.

Twenty tumor samples and ten paired adjacent normal tissues were snap-frozen after surgical resection and stored at -80 ℃. DNA was isolated from the tumor tissue by proteinase K digestion followed by phenol-chloroform extraction according to standard protocols.

Array CGH assay

Array CGH was performed according to the manufacturer's protocol with minor modifications on a 385k oligonucleotide chip (Roche/NimbleGen Systems Inc., Madison, WI). Commercially available pooled normal control DNA (Promega Corporation, Madison, WI) was used for reference. The patient DNA and the reference DNA were labeled with either cyanine 3 (Cy-3) or cyanine 5 (Cy-5) by random priming (Trilink Biotechnologies, San Diego, CA) and then hybridized to the chip *via* incubation in the MAUI hybridization system (BioMicro Systems, Salt Lake City, UT). After 18-h hybridization at 42 ℃, the slides were washed and scanned using a GenePix 4000B (Molecular Devices, Sunnyvale, CA).

Statistical analysis

Profile smoothing and breakpoint detection were performed with NimbleScan version 2.4 and SignalMap version 1.9 (NimbleGen Systems). If a smoothed copy number $\log_2$ ratio was above 0.15 or below -0.15 across five neighboring probes, it was defined as a gain or a loss, respectively. Amplifications were defined as those with a smoothed DNA copy number ratio above 0.5.

RESULTS

Overview of genomic imbalances in 20 primary gastric adenocarcinomas

An overview of genomic imbalances in the twenty advanced primary gastric adenocarcinomas is shown in Figure 1. Genomic copy number changes (gains, losses, amplifications or homozygous deletions) were detected in all cases except one. Net gains (15 cases) of genetic material were more frequent than net losses (4 cases). The sizes of the net genomic imbalances per sample ranged from a loss of 122.2 Mb (4.1% of genome) to a gain of 336.9 Mb (11.2% of genome) (Table 1). The mean number of gains per case was 9.0, ranging from 0 to 40, and the mean number of losses per case was 3.5, ranging from 0 to 14. The gain sizes ranged from 56.3 kb to 158.6 Mb, and the loss sizes ranged from 150.1 kb to 131 Mb. Approximately 28% (70/250) of the genomic imbalances were smaller than 1 Mb; from this subset, 21.6% (54/250) of the total imbalances were gains, and 6.4% (16/250) were losses. The most frequent

Table 1 Clinical characteristics, risk factors and overall genomic imbalances in 20 gastric adenocarcinomas

No.	ID	Sex/age (yr)	T/N/M stage	Tumor type	Histology grade (differentiated)	Tumor location	Smoke history	Drink history	Genomic size of total gain (Mb)	Genomic size of total loss (Mb)	Net imbalances (Mb) (%)[2]
1	T64	M/75	T3/N3/M0	Tubular	Poorly	Upper (CR), lower (AA)	Y	Y	290.7	147.3	+143.4 (4.8)
2	TW0800	M/65	T3/N3/M0	Tubular	Poorly	Upper (CR/GF), central (GB)	N	N	388.9	205.3	+183.6 (5.6)
3	T74	M/58	T3/N3/M0	Tubular	Poorly	Central (GB)	N	Y	290.1	412.3	-122.2 (4.1)
4	TW0784	M/75	T3/N1/M0	Tubular	Poorly	Upper (CR/GF)	N	N	3.4	0	+3.4 (0.1)
5	T66	M/50	T3/N2/M0	Tubular	Poorly	Lower (AA)	N	Y	34.2	0	+34.2 (1.1)
6	T78	F/59	T3/N1/M0	Tubular	Poorly	Lower (AA)	N	N	146.2	0	+107.4 (3.6)
7	T41	M/73	T3/N2/M0	Tubular	Poorly	Lower (AA/P)	Y	Y	7.2	0	+7.2 (0.2)
8	T47	F/52	T3/N1/M0	Tubular	Poorly	Lower (AA)	N	N	0.0	0.0	0.0 (0.0)
9	T38	F/53	T2/N1/M0	Tubular	Poorly	Lower (AA/P)	N	N	294.3	37.4	+256.9 (8.6)
10	TW0796	M/56	T3/N1/M0	Tubular	Poorly	Lower (AA/P)	Y	Y	224.4	11.9	+212.5 (7.1)
11	TW0807	M/54	T3/N0/M0	Tubular	Poorly	Lower (AA/P)	Y	Y	191.7	43.7	+148.0 (4.9)
12	TW0813	F/57	T3/N2/M0	Tubular[1]	Poorly	Lower (AA/P)	N	N	76.1	54.6	+21.5 (0.7)
13	T52	M/73	T3/N2/M0	Tubular	Moderately	Upper (CR/GF)	N	N	333.9	361.8	-27.9 (0.9)
14	TW0797	M/62	T3/N1/M0	Tubular	Moderately	Upper (CR/GF)	N	Y	161.1	0	+161.1 (5.4)
15	TW0782	M/53	T3/N2/M0	Tubular	Moderately	Upper (CR/GF)	Y	N	290.1	108.3	+181.8 (6.1)
16	TW0780	F/59	T2/N0/M0	Tubular	Moderately	Lower (AA/P)	N	N	4.1	4.16	-0.06 (0.0)
17	T75	M/69	T3/N2/M0	Tubular	Moderately	Lower (AA)	Y	Y	28	0	+28.0 (0.9)
18	T76	F/59	T3/N1/M0	Mucinous	Poorly	Upper (CR/GF)	Y	Y	99.1	0	+99.1 (3.3)
19	TW0789	M/64	T3/N1/M0	Mucinous	Poorly	Lower (AA/P)	N	N	504.5	167.6	+336.9 (11.2)
20	TW0774	F/76	T3/N3/M0	Mucinous	Poorly	Lower (AA/P)	N	N	41.4	90.4	-49.0 (1.6)

[1]Signet ring cell; [2]Percent of net imbalances calculated based on 3000 Mb of genome size. F: Female; M: Male; T: Tumor; N: Node; M: Metastasis ; CR: Cardiac region; GF: Gastric fundus; GB: Gastric body; AA: Antral area; P: Pylorus; Y: Yes; N: No.

genomic imbalances detected in these cancer samples were gains of 3q26.31-q27.2 (6/20), 5p (5/20), 8q [12/20: 8q22.2-q22.3 (10), 8q24.13-q24.22 (12)], 11p (6/20), 18p (4/20), 19q (8/20) and 20q (8/20) and losses of 3p14.2 (6/20), 4p15.1 (6/20), 18q21.2-q22.1 (4/20) and 21q21.1-q21.2 (4/20). However, no genomic imbalances were detected in the ten paired adjacent tissues, demonstrating that these genomic imbalances are tumor related.

Genomic regions with amplification: Possible diagnostic marker loci

The most prominent feature in this study was the amplicons of 11q13 (two tubular and one mucinous), 18q11.2 (three tubular) and 19q12 (three tubular), as well as the novel amplicons 1p36.22 (two tubular) and 11p15.5 (three tubular) (Table 2 and Figure 2). Amplification of 11q13 had the smallest region of overlap (SRO) of 343.7 kb, which included the *CCND1*, *ORAOV1* and *FGF19* genes. Amplification of 18q11.2 had an SRO of 625.0 kb, which included the *GATA6* and *CTAGE* genes. Amplification of 19q12 had an SRO of 1.4 Mb, which included nine genes: *LOC148145*, *LOC10050583*, *UQCRFS1*, *LOC284395*, *VSTM2B*, *POP4*, *PLEKHF1*, *C19orf12* and cyclin E1 (*CCNE1*). The SRO of the novel amplification 1p36.22 was 418.7 kb and included *FEX14*, *CASZ1*, *C1orf127* and *TARDBP*. The SRO of the other novel amplification, 11p15.5, was 343.7 kb and included *MUC5B*, *LOC255512*, *TOLLIP*, *BRSK2*, *HCCA2*, *DUSP8* and *LOC338865*. Other regions in which amplification was detected were 8p23.1, 8q24.21, 10q26.12q26.13, 11q13, 12p12.1, 12q15, 17q12 and 20q13.2; these regions are summarized in Table 2.

The most common losses involved the fragile histidine triad/FRA3B and PDCH7 genes

Six cases had a loss of the fragile histidine triad (*FHIT*) gene, which maps to the common fragile site 3p14.2. Five of these were intergenic losses of the *FHIT* gene,

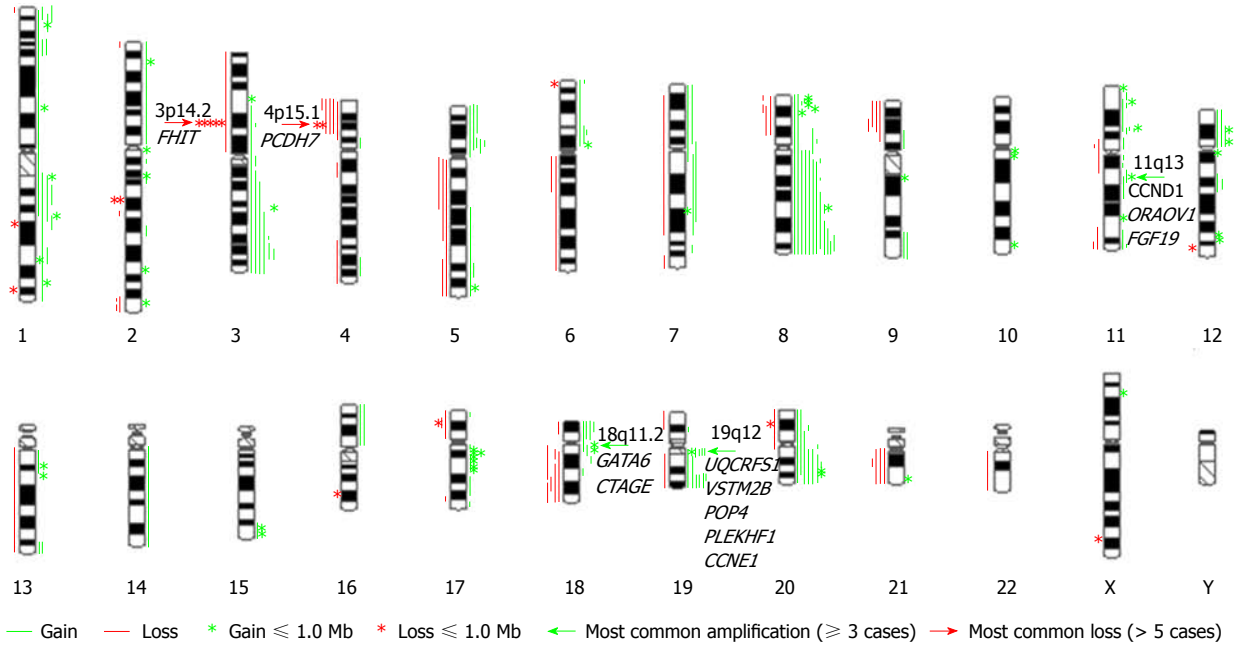

Figure 1 An overview of genomic imbalances in 20 primary gastric adenocarcinomas. The total number of gains was 180 (54 ⩽ 1 Mb), and the total number of losses was 70 (16 ⩽ 1 Mb). Partial or whole gains of 8q (12/20), 19q (8/20), and 20q (8/20) were most frequent. The most common amplicons were of the 11q13, 18q11.2 and 19q12 regions (green arrows). The loss of the *FHIT* gene and the partial loss of 4p with the smallest region of overlap including the *PCDH7* gene were the most frequent losses (red arrow).

and one of these five cases (T38) showed a homozygous deletion. The sizes of these losses ranged from 88.3 kb to 762.5 kb (Table 3 and Figure 3). Another common loss identified in all six cases was the 4p15.1 region (Table 3 and Figure 3). Two cases (TW789 and TW782) had an intergenic loss of the *PDCH7* gene.

DISCUSSION

In this study, we investigated gene/segmental genomic copy number alterations in twenty advanced primary gastric adenocarcinomas (seventeen tubular and three mucinous) *via* whole-genome array CGH. We observed that the total number of gains/amplifications (180) was 2.6 times the total number of losses (70). Nineteen out of the 20 cases had genomic imbalances; fifteen of these had net genomic gains (3.4-336.9 Mb), and four of these had net genomic losses (0.06-122.2 Mb), indicating that genomic gains are more common than losses (Figure 1). These findings are compatible with previous findings determined *via* conventional CGH[7-51]. However, we discovered cryptic genomic imbalances smaller than 1.0 Mb in 28% (70/250) of the total imbalances (with an average of 3.5 per case) and narrowed down the SROs of losses or of gains/amplifications to those including interesting genes or focal genomic segments. These findings are explained by the resolution of the array that we used. The most interesting cryptic imbalances were losses of 3p14.2 and 4p15.1 (Figure 3) and amplifications of 1p36.22, 11p15.5, 11q13, 18q11.2 and 19q12 (Figure 2).

The loss of 3p14.2 in six of our cases encompassed the *FHIT* gene, which was discovered at the FRA3B locus on the short arm of chromosome 3 and is the most active common chromosome fragile site in the human genome. *FHIT*'s loss of function as a tumor suppressor gene due to breakage, allelic loss, occasional homozygous deletion and promoter hypermethylation has been evaluated in different types of epithelial tumors, including gastric cancer, which is strongly associated with direct or indirect exposure to environmental carcinogens[52-54]. The FHIT protein is absent in more than 50% of the observed cases, both in gastric cancer cell lines and in primary gastric adenocarcinomas, irrespective of any specific histotype, indicating that alterations of the *FHIT* gene can play a role in tumorigenicity as an important and preliminary genetic alteration in the cell[55]. However, subsequent additional genetic changes involving other tumor suppressor genes or oncogenes may be necessary for tumor progression. In our six cases who had lost the *FHIT* gene, five showed an intergenic loss of *FHIT*, ranging in size from 150 kb to 762.4 kb. One case had a homozygous deletion of part of the *FHIT* gene (case T36); however, no deletion of this region was found in the normal adjacent tissue of the same case (date not shown). This finding suggests that *FHIT* loss, not normal copy number variation, is clearly linked to tumorigenicity. However, copy number variations, particularly losses including the *FHIT* gene, have been reported in the normal population[56], raising the question of whether constitutional copy number loss of the *FHIT* gene increases tumorigenicity susceptibility.

The other most common loss was of 4p15.1, detected in six cases and encompassing the *PDCH7* gene. PDCH7 is an integral membrane protein that is thought

Table 2 Amplification segments and the genes involved

Chr. region	Amp	Gain	SRO, bp (hg 18)	Size (kb)	Genes	Selected references
1p36.22[1]	2	1	10 587 540-11 006 299	418.7	*PEX14, CASZ1, C1orf127, TARDBP*	N/A
1q21.2	1	3	148 737 723-149 062 575	324.9	*TARS2, ECM1, ADAMTSL4*[3], *MCL1*[3], *ENSA*[2], *GOLPH3L, HORMAD1*[3], *CTSS, CTSK, ARNT*[2]	Gastric cancer[7], adenocarcinoma of the gastroesophageal junction[8][3], basal/luminal breast cancer[9][3], hepatocellular carcinoma[10]
8p23.1	2	3	10 475 239-10 562 632	87.4	*RP1 L1*	Gastric cancer[7,11], esophageal adenocarcinoma[12,13][3],[14],[15][3], adenocarcinomas of the gastroesophageal junction[16][3], small bowel adenocarcinoma[17]
			10 681 251-10 943 920	262.7	*PINX1, XKR6*	
			11 250 228-11 887 602	637.4	*TDH, FAM167A, BLK, GATA4*[2,3,4], *NEIL2*[2], *FDFT1*[2,3], *CTSB*[2,3]	
8q24.21	1	11	128 331 422-128 837 626	506.2	*POU5F1B, LOC727677, MYC*[2,3]	Various cancer[5], esophageal adenocarcinoma[14], gastric cancer[18],[19][2],[20], papillary renal cell carcinoma[21][3]
10q26.12	1	0	121 881 486-123 931 430	2050.0	*PPAPDC1A, LOC283089, WDR11, FGFR2*[3], *ATE1*[3], *NSMCE4A*[3], *TACC2*[3]	Breast cancer[22][3], gastric carcinoma[20]
10q26.13			126 212 750-126 362 686	149.9	*LHPP, FAM53B*	
11p15.5[1]	1	0	1 175 114-1 556 281	381.2	*MUC5B, LOC255512, TOLLIP, BRSK2, HCCA2, DUSP8, LOC33865*	N/A
11p13	2	1	34 675 094-35 068 916	393.8	*APIP*[3], *PDHX*[3]	Breast cancer[23], gastric cancer cell line[24], head and neck squamous cell carcinoma cell line[25][3]
11q13.2q13.3	3	0	68 912 663-69 256 388	343.7	*CCND1*[2,3], *ORAOV1*[2,3], *FGF19*[3]	Various cancers[5], gastric cancer[7,11,20],[26][2], hepatocellular carcinoma[27][2], esophageal adenocarcinoma[14], esophageal and gastric cancer[26], esophageal squamous cell carcinoma[28][3], laryngeal/pharyngeal cancer[29][3]
12p12.1	2	2	25 150 060-25 437 736	287.7	*LRMP*[3], *CASC1, LYRM5, KRAS*[2,3]	Various cancers[5], esophageal adenocarcinoma[14], gastric cancer[11,20],[30][2], ovarian cancer[31][3]
12q15	2	0	67 475 003-67 875 102	400.1	*MDM2*[2], *CPM*	Various cancers[5], esophageal adenocarcinoma[14], gastric cancer[20,32], liposarcomas[33][3], melanoma cell line[34][3]
			68 037 717-68 318 830	281.1	*YEATS4 (GAS41)*[3], *FRS2*[3], *CCT2*[3], *LRRC10*	
17q12	2	1	35 000 176-35 150 077	149.9	*NEUROD2, PPP1R1B*[2], *STARD3*[2], *TCAP, PNMT*[2], *PERLD1*[2], *ERBB2*[2], *C17orf37, GRB7*[2]	Various cancers[5], gastric cancer[11,20],[35-37][2],[38],[39][2]
18q11.2	3	2	17 800 202-18 425 167	625.0	*GATA6*[3,4], *CTAGE1*	Pancreatic carcinoma[40,41][3], esophageal adenocarcinoma[14],[42][3]
19q12	3	3	33 606 476-35 037 396	1340.9	*LOC148145, LOC10050583, UQCRFS1*[2], *LOC284395, VSTM2B, POP4*[2], *PLEKHF1*[2], *C19orf12*[2], *CCNE1*[2]	Gastric cancer[43][2],[44],[45][2], esophageal/gastric cardiac adenocarcinoma[12]
20q13.2	1	6	51 568 862-51 993 797	424.9	*ZNF217*[3], *SUMO1P1, BCAS1*[3]	Esophageal adenocarcinoma[14], adenocarcinoma of the gastroesophageal junction[46][3], breast cancer[47], gastric adenocarcinoma[48], glioblastoma[49], various cancers[50]

[1]Novel amplicon; [2]Gene and references that are overexpressed when amplified in gastric cancer; [3]Genes (and references) that are overexpressed when amplified in types of cancer other than gastric cancer; [4]Underexpression has been reported in gastric cancer. SRO: Smallest region of overlap; N/A: Not available.

to function in cell-cell recognition and adhesion. Loss of heterozygosity (LOH) or deletion of this region has been reported in hepatocellular carcinoma (HCC) and in some head and neck squamous cell carcinomas[57,58]. Recently, a genome-wide analysis revealed a single-nucleotide polymorphism in *PDCH7* whose risk allele affects overall survival in early-stage non-small-cell lung cancer[59].

Gene/chromosomal segment amplifications are thought to reflect genetic instabilities in solid-tumor cells[60]. Such amplifications commonly consist of double minutes (DMs) or homogeneous staining regions or are dispersed at the genomic level; they are usually correlated with protein levels of genes[61]. It has been proposed that the activation of proto-oncogenes by amplification plays an important role in the development of many human solid tumors. Therefore, detection of specific gene amplifications in tumor cells can lead to the identification of genes putatively involved in growth control and tumorigenesis. In our study, we identified the novel amplicons 1p36.22 and 11p15.5 as well as prominent amplicons 11q13, 18q11.2 and 19q12 (≥ 3 cases with amplification).

LOH or the loss of the short arm of chromosome 1, which includes band p36, has been reported in vari-

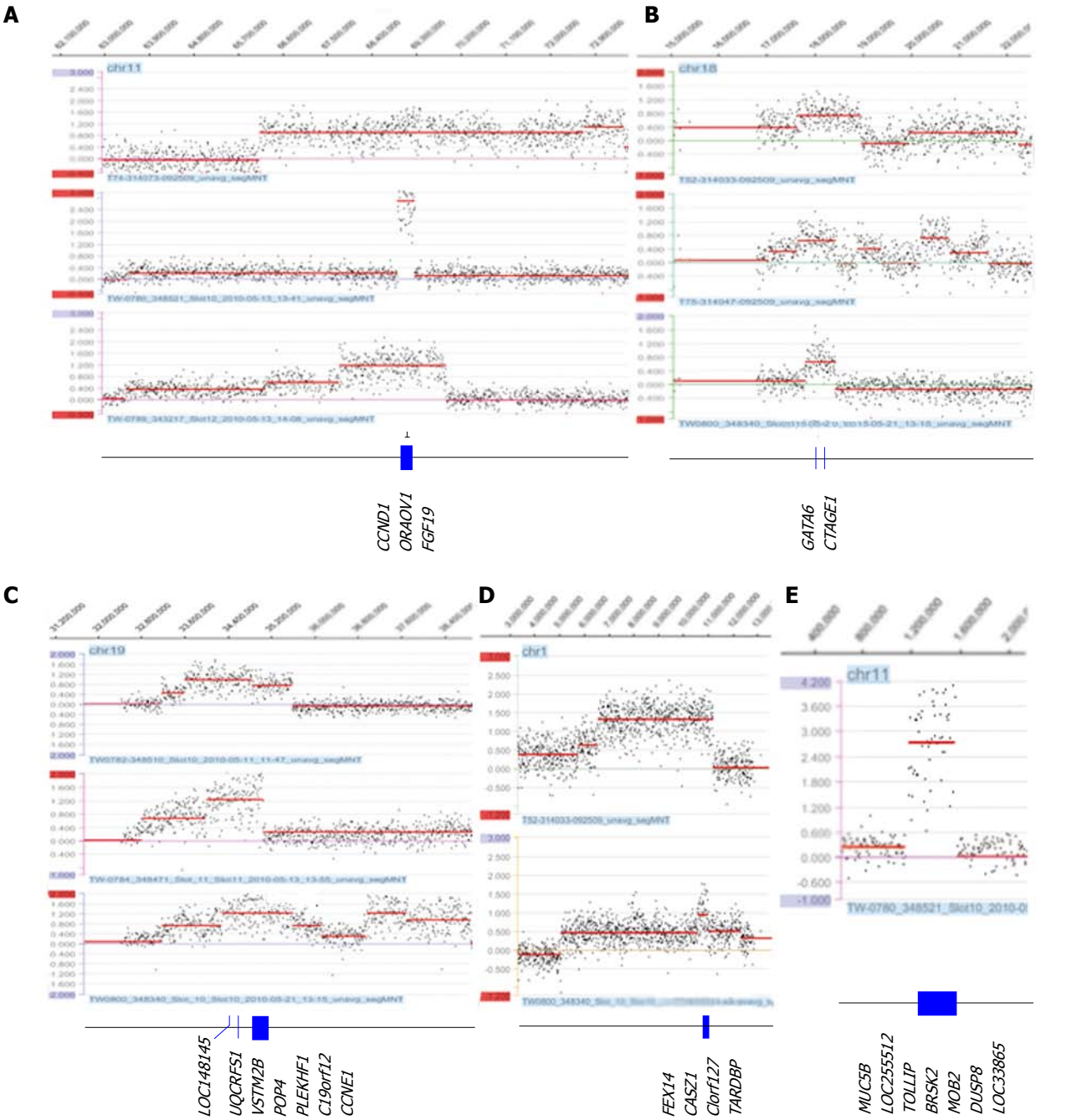

Figure 2 Representative amplifications detected by array comparative genomic hybridization and the genes that are located in the smallest region of overlap. The most common amplicons at 11q13 (A), 18q11.2 (B) and 19q12 (C) and novel amplicons at 1p36.22 (D) and 11p15.5 (E) region (log2 > 0.5). The x-axis indicates the genomic location, and the y-axis indicates the log2 ratio.

ous cancers[62], supporting the possibility that this region encompasses at least one tumor suppressor gene, as opposed to one or more oncogenes. The *CASZ1* gene on 1p36, which was amplified in our cases, has been implicated as a candidate tumor suppressor gene in neuroblastomas[63]. However, our study revealed three cases with a gain of 1p36.22. Of these, two cases with high-level amplification of a 418.7 kb SRO, which includes the *PEX14*, *CASZ1*, *C1orf127* and *TARDBP* genes, had not previously been reported. Further studies could determine whether this amplification induces overexpression of proteins. The other novel amplicon, found in one case, was the 11p15.5 region, which is 343.7 kb and includes the *MUC5B*, *TOLLIP*, *BRSK2*, *HCCA2*, *DUSP8* and *LOC33865* genes. The *MUC5B* gene encodes a member of the mucin family of proteins, which are highly glycosylated macromolecular components of mucus secretions. This family member is the major gel-forming mucin in mucus. The expression of this gene has been associated with a type of gastric carcinoma but not with the clinical-biological behavior of the tumors[64,65]. The *TOLLIP* gene encodes a ubiquitin-binding protein that interacts with several toll-like receptor (TLR) signaling cascade components. The TOLLIP

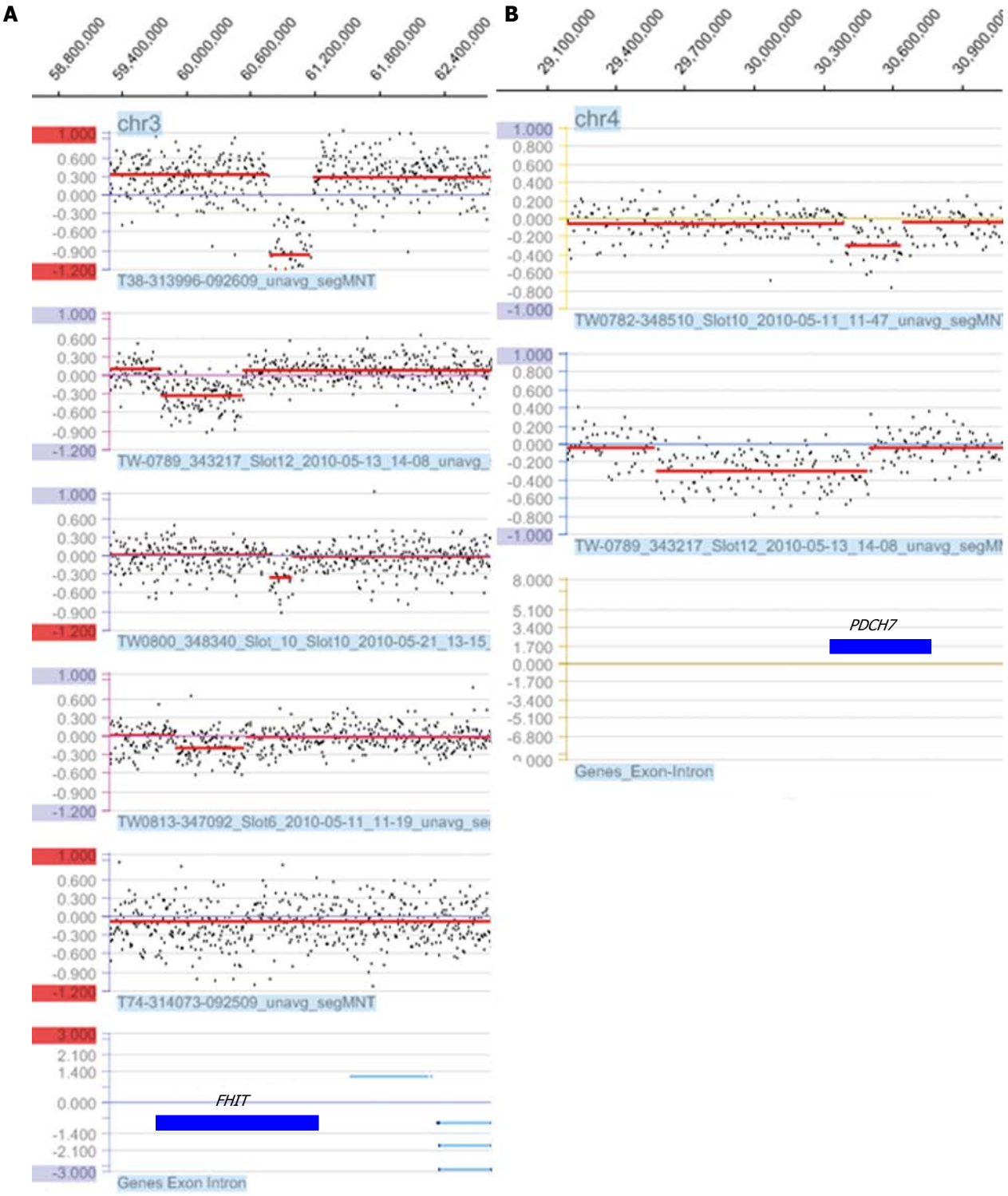

Figure 3 Intergenic loss of *FHIT*/FRA3B on 3p14.2 (A) and *PDCH7* on 4p15.1 (B) detected by array comparative genomic hybridization. The x-axis indicates the genomic location, and the y-axis indicates the log2 ratio.

protein regulates inflammatory signaling and is involved in interleukin-1 receptor trafficking and the turnover of the IL1R-associated kinase; a possible association with human cancer development has been suggested[66]. The BR serine/threonine kinase 2 (*BRSK2*) gene acts as a checkpoint kinase upon DNA damage induced by UV irradiation or methyl methane sulfonate[67]. Clinical implications of *BRSK2* expression in pancreatic ductal adenocarcinoma have been suggested[68]. The hepatocellular carcinoma-associated gene 2 (*HCCA2*) gene was initially identified as a HCC-specific protein. It was subsequently found to interact with *MAD2L2* and might function in cell cycle regulation[69]. The dual specificity phosphatase 8 (*DUSP8*) gene is a member of the *DUSP* subfamily.

Table 3 Summary of losses of *FHIT* at 3p14.2 and of *PDCH7* at 4p15.1

Gene	Chromosome region	Case	Genomic coordinates (NCBI build 36.3; hg18)	Size (kb)
FHIT (HD)	3p14.2	T38	60 775 135-61 162 550	387.4
FHIT	3p14.2	TW0789	59 762 657-60 525 130	762.5
FHIT intron	3p14.2	TW800	60 781 394-60 987 631	206.2
FHIT	3p14.2	TW813	59 900 085-60 543 794	437.5
FHIT etc.	3pter-p11.2	T74	37 570-88 331 466	88 300.0
FHIT intron	3p14.1	T782	60 343 805-60 493 871	150.1
PDCH7 etc.	4p16.3-p13	T64	2 431 351-44 331 469	41 900.0
PDCH7	4p15.1	TW789	29 575 024-30 487 701	912.7
PDCH7 etc.	4pter-p14	TW807	191-37 525 019	37 500.0
PDCH7 etc.	4pter-p14	TW774	191-38 356 422	38 400.0
PDCH7	4p15.1	TW782	30 393 830-30 637 746	243.9
PDCH7 etc.	4pter-p14	TW813	191-38 993 759	39 000.0

HD: Homozygous deletion.

DUSPs inactivate their target kinases by dephosphorylating both the phosphoserine/threonine and phosphotyrosine residues. These genes negatively regulate members of the mitogen-activated protein kinase (MAPK) superfamily (MAPK/ERK, SAPK/JNK, p38), which are involved in cellular proliferation and differentiation. The roles of the DUSPs in the regulation of MAPK activities have been highlighted as part of the oncogenic process[70]. Overall, any of the genes in this region are likely to play an important role in the progression of cancer.

The amplification of 11q13 ranged from 343.7 kb to 20.5 Mb and had an SRO of 343.7 kb, which included the *CCND1*, *ORAOV1* and *FGF19* (*BLC6*) genes. The variously sized 11q13.3 amplicon containing *CCND1* is one of the most frequent amplification events in human tumors. Computational genome-wide approaches to identify driver genes have reported *CCND1* as one of the most common somatic focal amplifications in human cancers[5]. As shown in our 11q13 amplification, the *ORAOV1* and *FGH19* genes lie within 15 kb of *CCND1*, and they are invariably co-amplified with *CCND1*. However, the limited data show that their expression levels depend on the type of tumor, indicating that the driver gene can be tissue-type dependent/specific. The co-expression of *FGH19* with or without *CCND1* has been found in HCC, but an absence of *FGH19* expression has been found in breast cancer and oral cancer[27]. Future work can determine whether *FGH19* is co-expressed with *CCND1* in primary gastric adenocarcinoma, even given that recently published whole-genome expression data did not show a significant correlation or co-expression of amplification/expression of *CCND1* and *FGH19*[35,43]. *ORAOV1* overexpression has been found in all amplified HCCs; however, *ORAOV1* does not promote tumorigenicity in $p53^{-/-}$; Myc hepatoblasts, nor is it cooperative with *FGFR1* or *CCND1*[27]. A total of four cases in this study had a gain of the 18q11.2 region. Of these, three cases had amplifications ranging from 625.0 kb to 1.3 Mb with an SRO of 625.0 kb, which includes the *GATA6* and *CTAGE* genes. This 18q11.2 amplification, along with expression and epigenetic studies, supports the oncogenic function of *GATA6* in esophageal carcinoma, colon cancer and pancreatic cancer[40,42,71,72] but not in gastric adenocarcinoma. Upregulation of *GATA6* has been reported in the transition from normal esophageal epithelium to Barrett adenocarcinoma and adenocarcinoma[42,71]. A 0.36 kb amplification including *GATA6* and *CTAGE1* has been found in pancreatic carcinoma[40,41], and dysregulation (overexpression) of *GATA6* contributes to colorectal tumorigenesis and tumor invasion[72]. Moreover, in that study *GATA6* was overexpressed not only in the cases with an amplification of *GATA6* but also in the cases without amplification[72]. However, *CTAGE* was rarely overexpressed, indicating that *GATA6* is the driver in this amplicon[41] and suggesting that *GATA6* overexpression may play a role in the early stages of tumor development. A contradictory result showing underexpression of the *GATA6* gene has been reported in gastric adenocarcinoma[36], demonstrating that the oncogenic function of *GATA6* in gastric adenocarcinoma needs to be investigated. Our observed amplification of 19q12 with an SRO of 1.4 Mb includes the nine genes *LOC148145*, *LOC10050583*, *UQCRFS1*, *LOC284395*, *VSTM2B*, *POP4*, *PLEKHF1*, *C19orf12* and *CCNE1*. This 19q12 amplification has been found in gastric cancer and esophageal adenocarcinoma[12,44,45]. Of these genes, *CCNE1*, an E type cyclin, has traditionally been considered the target of the 19q amplification, which is also one of the most common amplification products in various tumor types[5,12]. However, a comprehensive analysis of the 19q12 amplification in gastric cancer has revealed clustered overexpression of *CCNE1* as well as *UQCRFS1*, *POP4*, *C19orf12* and *RMP*, indicating potential functions of other genes in this region in tumor development[45]. In ovarian cancer, it has been suggested that the *CCNE1* gene is the key driver in this 19q12 amplicon and is correlated with the gain in the 20q11 region that includes the *TPX2* gene[73]. It is not clear whether the *CCNE1* gene is a key driver in other types of tumors, including gastric cancer tumors, because no detailed study has been conducted; it is also possible that this gene is tissue specific, as shown in 19q12. However, the gain of the 20q11 region is one of the most

common findings in gastric cancer, and the correlation between 19q12 and 20q11 in gastric cancer needs to be investigated.

Other amplifications found were 1q21.2 (1 amp/3 gains), 8p23.1 (2 amps/3 gains), 8p23.1 (2 amps/3 gains), 8q24.21 (1 amp/11 gains), 10q26.12q26.13 (1 amp), 11p13 (2 amps/1 gain), 12p12.1 (2 amps/2 gains), 12q15 (2 amps), 17q12 (2 amps/1 gain) and 20q13.2 (1 amp/6 gains). We summarized these amplifications and the expression of the gene(s) corresponding to these amplicons in various primary epithelial tumor and cell lines (Table 2). *MCL1* on 1q21.1, *MYC* on 8q24.21, *KRAS* on 12p12.1, *MDM2* on 12q15, *ERBB2* on 17q12 and *ZNF217* on 20q13.2 are well-acknowledged oncogenes in several tumors[5]. Recently published data suggest that the same amplifications do not necessarily induce the same gene expression in different tissue types[27], implying that driver genes can be tissue-type specific and making it necessary to acquire and investigate both amplification and overexpression information for different tumor types, even in the case of well-validated oncogenes.

In summary, the array CGH technique allows for comprehensive, rapid and reliable analysis of the whole genome of primary gastric adenocarcinomas and enables the refined and detailed study of amplifications and regions of recurrent copy number change. This approach makes it possible to identify putative oncogenes and tumor suppressor genes that may deserve further investigation. In this context, we identified candidate target genes/genomic segments of amplification that may help to direct therapeutics against gastric cancer.

COMMENTS

Background

Gastric cancer is one of the leading causes of cancer-related death worldwide. Although its incidence has gradually decreased in many Western countries, the incidence of gastric cancer still remains high in South and Central America and is highest in Eastern Asia, specifically in China, South Korea and Japan. Searching for biomarkers for gastric cancer has proven to be quite challenging.

Research frontiers

Cancer is a genetic disease that involves multiple genetic alterations. Understanding the molecular profile of tumor tissue is crucial for efficiently targeting cancer cells. In this study, the authors identified possible candidate target genes that could provide insight into the pathogenesis of gastric adenocarcinoma through the integration of genomic copy number changes.

Innovations and breakthroughs

Recent reports have highlighted the oncogenic addiction associated with oncogene amplification as a driver gene in carcinogenesis and target treatments. In this research paper, the authors identified novel amplicons that have not been published previously and that could be new target sites for potential diagnosis, prognosis and treatment.

Applications

To learn how these new amplicons are induced and change gene expression, this study may represent a future strategy for therapeutic intervention in the treatment of patients with gastric adenocarcinoma.

Peer review

The paper was designed to be a proof of principle for using cytogenomic microarrays on a larger study to ascertain biomarkers for various gastric neoplasms. The authors did a very thorough analysis of the genetic alterations and of the discussion of the possible genes involved. As claimed, they showed the power of the cytogenomic microarray in detecting common genetic alterations in various gastric tumors. The study was organized in a very clear manner and each common abnormality was compared in a thoughtful manner.

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BRIEF ARTICLE

FOLFIRI regimen in metastatic pancreatic adenocarcinoma resistant to gemcitabine and platinum-salts

Cindy Neuzillet, Olivia Hentic, Benoît Rousseau, Vinciane Rebours, Léïla Bengrine-Lefèvre, Franck Bonnetain, Philippe Lévy, Eric Raymond, Philippe Ruszniewski, Christophe Louvet, Pascal Hammel

Cindy Neuzillet, Olivia Hentic, Vinciane Rebours, Philippe Lévy, Philippe Ruszniewski, Pascal Hammel, Service de Gastroentérologie-Pancréatologie, Hôpital Beaujon, 100 Boulevard du Général Leclerc, 92110 Clichy La Garenne, AP-HP, France
Cindy Neuzillet, Benoît Rousseau, Léïla Bengrine-Lefèvre, Service d'Oncologie Médicale, Hôpital Saint Antoine, 184 Rue du Faubourg Saint-Antoine, 75012 Paris, AP-HP, France
Franck Bonnetain, Unité de Biostatistique et d'Epidémiologie EA 4184, Centre Georges François Leclerc, 1 Rue du Pr Marion, 21079 Dijon, France
Eric Raymond, Service d'Oncologie Médicale, Hôpital Beaujon, 100 Boulevard du Général Leclerc, 92110 Clichy La Garenne, AP-HP, France
Christophe Louvet, Département d'Oncologie Médicale, Institut Mutualiste Montsouris, 42 Boulevard Jourdan, 75014 Paris, France
Author contributions: Neuzillet C, Louvet C and Hammel P designed the study; Neuzillet C, Hentic O, Rousseau B, Bengrine-Lefèvre L, Lévy P, Raymond E, Ruszniewski P, Louvet C and Hammel P performed the research; Rebours V and Bonnetain F analyzed the data; and Neuzillet C and Hammel P wrote the paper.
Correspondence to: Dr. Pascal Hammel, Service de Gastroentérologie-Pancréatologie, Hôpital Beaujon, 100 Boulevard du Général Leclerc, 92110 Clichy La Garenne, AP-HP, France. pascal.hammel@bjn.aphp.fr
Telephone: +33-1-40875653 Fax: +33-1-42703784
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Abstract

AIM: To evaluate the efficacy and safety of the FOLFIRI regimen in patients with metastatic pancreatic adenocarcinoma (PAC) after the failure of gemcitabine and platinum salts.

METHODS: All consecutive patients with histologically confirmed, metastatic PAC and World Health Organization performance status (PS) $\leqslant$ 2 received FOLFIRI-1 [irinotecan 180 mg/m^2 on day 1 and leucovorin 400 mg/m^2 followed by 5-fluorouracil (5-FU) 400 mg/m^2 bolus, then 5-FU 2400 mg/m^2 as a 46-h infusion, biweekly] or FOLFIRI-3 (irinotecan 100 mg/m^2 on day 1 and leucovorin 400 mg/m^2, then 5-FU 2400 mg/m^2 as a 46-h infusion and irinotecan 100 mg/m^2 repeated on day 3, biweekly) after failure of gemcitabine and platinum-based chemotherapies as a systematic policy in two institutions between January 2005 and May 2010. Tumor response, time to progression (TTP), overall survival rate (OS) and grade 3-4 toxicities were retrospectively studied. Subgroup analyses were performed to search for prognostic factors.

RESULTS: Sixty-three patients (52.4% male, median age 59 years) were analyzed. Among them, 42.9% were PS 0, 38.1% were PS 1 and 19.0% were PS 2. Fifty one patients (81.0%) had liver metastases. Before the FOLFIRI regimen, patients had received 1 line (n = 19), 2 lines (n = 39) or 3 lines (n = 5) of chemotherapy. Median TTP obtained with the line before FOLFIRI was 3.9 mo (95% CI: 3.4-5.3 mo). A total of 480 cycles was completed (median: 6 cycles, range: 1-51 cycles). The main reason for discontinuing FOLFIRI was tumor progression (90.3%). Tumor control was achieved in 25 patients (39.7%) (partial response: n = 5, stable disease: n = 20) with FOLFIRI. Median TTP was 3.0 mo (95% CI: 2.1-3.9 mo) and median OS was 6.6 mo (95% CI: 5.3-8.1 mo). Dose adaptation was required in 36 patients (57.1%). Fifteen patients (23.8%) had grade 3-4 toxicities, mainly hematological (n = 11) or digestive (n = 4). Febrile neutropenia occurred in 3 patients. There was no toxic death. PS 2 was significantly associated with poor TTP [hazard ratio (HR): 16.036, P < 0.0001] and OS (HR: 4.003, P = 0.004).

CONCLUSION: The FOLFIRI regimen had an acceptable toxicity and an interesting efficacy in our study, limited to patients in good condition (PS 0-1).

Key words: Pancreatic cancer; Pancreatic adenocarcinoma; Metastases; Chemotherapy; 5-fluorouracil; Irinotecan; Camptothecin; FOLFIRI regimen

Peer reviewer: Dr. Michel M Murr, MD, Director of Bariatric Surgery, Tampa General Hospital, 1 Tampa General Circle, Tampa, FL 33647, United States

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INTRODUCTION

Pancreatic adenocarcinoma (PAC) accounts for 2%-3% of all cancers but is the fourth leading cause of cancer death in Western countries[1]. More than 80% of patients present with unresectable disease and most of those with operable tumors who undergo resection have local relapse or metastases[2]. The overall prognosis of metastatic PAC remains poor, with a 5-year survival rate of less than 5%[3].

Gemcitabine became the reference regimen as first-line chemotherapy in patients with metastatic PAC after a randomized trial showed significant improvement in median overall survival (OS) compared with 5-fluorouracil (5-FU) (5.6 mo *vs* 4.4 mo, $P = 0.002$)[4]. Over the past decade, multiple phase Ⅱ and Ⅲ studies have attempted to improve these results using various combinations of gemcitabine with other agents but no significant benefit on survival has been found compared with gemcitabine alone, except for erlotinib which resulted in a modest but significant improvement in OS (6.2 mo *vs* 5.9 mo, $P = 0.038$)[5,6]. A phase Ⅲ trial comparing the FOLFIRINOX regimen (folinic acid/5-FU, irinotecan and oxaliplatin combination) to gemcitabine as first-line treatment for metastatic PAC showed that this combination was superior to gemcitabine (OS: 11.1 mo *vs* 6.8 mo, $P < 0.001$)[7].

In clinical practice, about half of metastatic PAC patients with disease progression under gemcitabine treatment remain in acceptable clinical condition and thus may receive subsequent line(s) of chemotherapy. A retrospective series of 117 patients evaluated the feasibility and benefits of second- and third-line chemotherapies in patients with metastatic PAC after the failure of gemcitabine[8]. Fifty three (45%) received two lines and 24 (21%) received three or more lines. Median time to progression (TTP) and OS from the beginning of the second line were 2.3 mo and 4.7 mo, respectively. The FFCD 0301 phase Ⅲ trial was the first randomized study to evaluate a chemotherapy strategy with a second line of treatment in the treatment plan. It compared the combination of folinic acid/5-FU and cisplatin followed by gemcitabine or the reverse sequence in metastatic PAC[9]. The second line of therapy was administered at disease progression to 68% of patients who received folinic acid/5-FU and cisplatin as a first line treatment and to 55% in the gemcitabine arm (non-significant). Median progression-free survival (PFS) and OS in the two arms were not significantly different. Although there is no standard regimen in this setting, two randomized studies have indicated that the combination of folinic acid/5-FU and oxaliplatin appeared to be superior to both best supportive care (4.9 mo *vs* 2.3 mo, $P = 0.008$) and folinic acid/5-FU alone (6.0 mo *vs* 3.0 mo, $P = 0.014$) as a second line of treatment[10]. Other regimens have been tested in non-randomized phase Ⅱ studies, but the samples were small and information on World Health Organization (WHO) performance status (PS) and disease stage were often lacking[11].

Preclinical studies have shown that the camptothecin analog irinotecan has significant activity in both cultured pancreatic tumor cells and in xenograft models[12,13]. Irinotecan monotherapy in previously untreated PAC patients yielded response rates (RR) of 9%-27%[14-16]. *In vitro* studies have suggested that there is a synergistic effect between irinotecan and 5-FU[17-19]. A multicenter phase Ⅱ study with folinic acid/5-FU and irinotecan day 1/day 3 combination (FOLFIRI-3 regimen) showed promising activity in chemotherapy-naive patients with locally advanced or metastatic PAC, with a median PFS and OS of 5.6 mo and 12.1 mo, respectively, and a manageable toxicity profile[20]. A randomized phase Ⅱ study has compared modified FOLFIRI-3 and a modified FOLFOX schema (folinic acid/5-FU and oxaliplatin combination) as second-line chemotherapy in that setting[21]. The efficacy was similar, with 6-mo OS rate of 27% and 30%, respectively. An Italian group reported a retrospective series of 40 patients with gemcitabine-resistant locally advanced or metastatic PAC treated with a standard FOLFIRI (FOLFIRI-1, folinic acid/5-FU and irinotecan day 1 combination) regimen[22]. Median TTP was 3.7 mo and median OS was 6 mo.

Because no data exist on the efficacy of the FOLFIRI regimen after the failure of both gemcitabine and platinum salts, we performed a retrospective study to evaluate the efficacy and safety of this regimen in patients with advanced PAC in that setting. As locally advanced PAC may have a more favorable natural history than metastatic PAC, we decided to exclude locally advanced PAC patients from the study to have a homogeneous population.

MATERIALS AND METHODS

Patients

All patients with histologically confirmed, metastatic PAC, after failure (progression or major toxicity) of gemcitabine and platinum-based chemotherapies, re-

ceived an irinotecan-based regimen as a systematic policy after discussion during a weekly multidisciplinary meeting in our institutions (Saint Antoine Hospital, Paris and Beaujon Hospital, Clichy), if they met the following criteria: previous treatment with gemcitabine and platinum salt (combined or given in consecutive lines); WHO PS ≤ 2; at least one bidimensionally measurable lesion according the Response Evaluation Criteria in Solid Tumors (RECIST); absence of severe uncontrolled cardiovascular, metabolic, infectious or renal disease; serum bilirubin level < 1.5 times the upper limit of normal; polynuclear neutrophil count > 1500/mm^3; platelet count > 100 000/mm^3.

Chemotherapy regimen

The FOLFIRI-1 regimen consisted of irinotecan 180 mg/m^2 administered as a 90-min infusion on day 1, together with leucovorin 400 mg/m^2 for 2 h followed by an 5-FU 400 mg/m^2 bolus, then a 46-h infusion of 5-FU 2400 mg/m^2. The FOLFIRI-3 regimen consisted of irinotecan 100 mg/m^2 administered as a 60-min infusion on day 1, together with leucovorin 400 mg/m^2 for 2 h, then a 46-h infusion (without bolus administration) of 5-FU 2400 mg/m^2 and irinotecan 100 mg/m^2 repeated on day 3 at the end of 5-FU infusion. Only FOLFIRI-3 (intensified) regimen has been evaluated in phase II studies in PAC[20,21]. However, the FOLFIRI-1 regimen is extensively used in clinical practice for treatment of other gastrointestinal cancers and seems to be less toxic. Thus, the choice between the FOLFIRI-1 or FOLFIRI-3 regimen was left up to the discretion of the investigator. The chemotherapy cycles were repeated every two weeks if the clinical and biochemical assessment was compatible (as mentioned above).

Patients who developed a cholinergic syndrome received preventive treatment with atropine (0.25 mg subcutaneously) during all subsequent cycles. Late-onset diarrhea was treated using high-dose loperamide. When severe neutropenia occurred and/or did not recover to grade ≤ 1 on day 14, a granulocyte-colony stimulating factor was given.

The irinotecan and the 5-FU dosages were reduced by 20% when any grade 3-4 toxicity occurred; other dose adjustments were decided on an individual basis. Treatment was stopped when the tumor progressed or severe toxicity occurred, or at the patient's request. Further treatments are discussed on an individual basis.

Assessment of therapeutic efficacy

Treatment efficacy was assessed on a clinical evaluation, carbohydrate antigen (CA) 19-9 serum levels and thoraco-abdominal computed tomography (CT). Assessment of treatment efficacy was performed every 2 mo (four cycles) or earlier in patients with clinically suspected progression. Tumor response was assessed using CT according to RECIST[23]. A complete response (CR) was defined as complete disappearance of all assessable disease, partial response (PR) as a decrease of > 30% in the sum of the largest diameters of target lesions, stable disease (SD) as a decrease of < 30% or an increase of < 20% in measurable lesions, and progressive disease (PD) as an increase of > 20% in measurable lesions or the appearance of new malignant lesions. Patients who were not assessable by CT but who presented clinical and/or biochemical (CA 19-9 serum level elevation) evidence of disease progression or who died from a cancer-related cause were also considered as PD. The sum of CR, PR and SD was reported as the tumor control rate (TCR). The sum of CR and PR was reported as overall RR (ORR). OS was defined as the time from the first day of the FOLFIRI regimen to the date of death (all causes) or last follow-up. TTP was defined as the time from the first day of the FOLFIRI regimen to the date of disease progression. Patients without progression were censored at the last follow-up.

Safety

Toxicity was assessed before each cycle with the National Cancer Institute Common Toxicity Criteria (version 3.0). A complete physical examination was performed and a full blood count and serum bilirubin, aminotransferases, alkaline phosphatase and creatinine assays were obtained before each treatment cycle.

Data collection

The following patient data were collected and analyzed retrospectively: age; gender; primary tumor location; stage at the time of diagnosis; previous surgery and/or radiotherapy; previous lines of chemotherapy; TTP with the previous line; reasons for stopping previous line; presence or absence of liver metastases at the beginning of FOLFIRI regimen; PS at the beginning of FOLFIRI regimen; type of FOLFIRI regimen (FOLFIRI-1 or FOLFIRI-3); number of cycles administered; best tumor response; grade 3-4 toxicities; dose adaptation; TTP and OS from the beginning of FOLFIRI regimen; reasons for stopping FOLFIRI regimen; further treatments.

Statistical analysis

All analyses were performed using Stata software (version 11.0; StataCorp). All statistical tests were two sided with an alpha type one error of 5%. TTP and OS were estimated using the Kaplan-Meier method and described using median or rate of TTP/OS at a specific time point with 95% CI. Log-rank tests were used to compare survival curves.

Univariate and multivariate Cox proportional hazard model analyses were performed with the following variables: stage at diagnosis; previous treatment by radiotherapy; number of previous chemotherapy lines; presence or absence of liver metastases; PS at the beginning of FOLFIRI regimen.

For exploratory purposes, subgroup analyses were performed according to the following variables: primary tumor location; stage at the diagnosis; previous treatment by surgery or radiotherapy; number of previous

Table 1 Characteristics of the 63 patients with metastatic pancreatic cancer treated with FOLFIRI after failure of gemcitabine and platinum-salts

Characteristics	Data
Age (yr)	
Median	59 (range: 24-81)
Sex (%)	
Male	33 (52.4)
Performans status (%)	
PS 0	27 (42.9)
PS 1	24 (38.1)
PS 2	12 (19.0)
Liver metastases (%)	
Present	51 (81.0)
Number of previous lines before FOLFIRI (%)	
1	19 (30.2)
2	39 (61.9)
⩾ 3	5 (7.9)

chemotherapy lines; presence or absence of liver metastases; PS at the beginning of FOLFIRI regimen; type of FOLFIRI regimen (FOLFIRI-1 or FOLFIRI-3).

RESULTS

Patients

Between January 2005 and May 2010, 63 patients with metastatic PAC fulfilled the criteria for this study. Their characteristics are shown in Table 1. Median age was 59 years (range: 24-81 years). Thirty three patients (52.4%) were male. The primary tumor was located in the head of the pancreas in 32/50 patients (64.0%). Twenty three patients (36.5%) had undergone prior surgery and 16 patients (25.4%) had received chemoradiotherapy. Twenty-seven patients (42.9%) were WHO PS 0, 24 patients (38.1%) were PS 1 and 12 patients (19.0%) were PS 2 at the beginning of the FOLFIRI regimen. Fifty one patients (81.0%) had liver metastases.

Before receiving the FOLFIRI regimen, patients had received one line (gemcitabine-oxaliplatin: n = 19, 30.2%), two lines (gemcitabine then FOLFOX regimen: n = 39, 61.9%) or three lines (n = 5, 7.9%) of chemotherapy. The previous line had been stopped for tumor progression in 55 patients (87.3%) and due to toxicity (oxaliplatin-related neuropathy) in the remaining 8 patients (12.7%).

Study treatment and drug delivery

Fifty five patients (87.3%) received the FOLFIRI-1 regimen and 8 patients (12.7%) received the FOLFIRI-3 regimen. A total of 480 cycles was completed (median: 6 cycles per patient, range: 1-51 cycles per patient).

The reasons for discontinuing the FOLFIRI regimen was progression in 56/62 patients (90.3%), toxicity in one patient (febrile neutropenia: n = 1, 1.6%), a tumor control ⩾ 6 mo or at the patient's request in 4 patients (6.5%), surgery in one patient (1.6%) who had a major response. Sixteen patients (25.4%) who remained in good condition at the time of FOLFIRI withdrawal received a subsequent line following the multidisciplinary proposal.

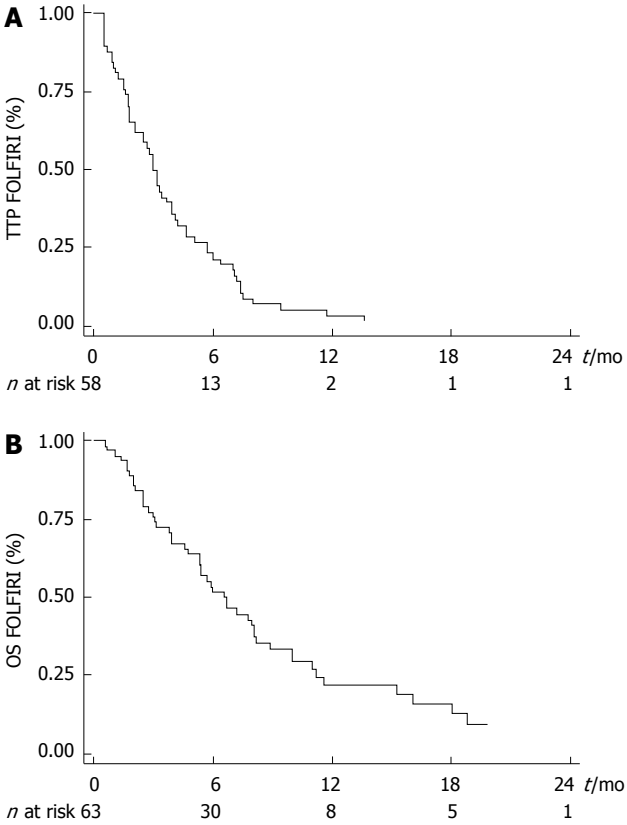

Figure 1 Time to progression (A) and overall survival (B) from the beginning of FOLFIRI for the 63 patients. TTP: Time to progression; OS: Overall survival.

Tumor response and survival

Median TTP obtained with the last line of chemotherapy before FOLFIRI was 3.9 mo (95% CI: 3.4-5.3 mo). Tumor control was obtained with the FOLFIRI regimen in 25 patients (39.7%) (CR: n = 0; PR: n = 5, 7.9%; SD: n = 20, 31.8%). ORR was 7.9% (5/63). Median TTP was 3.0 mo (95% CI: 2.1-3.9 mo) and median OS was 6.6 mo (95% CI: 5.3-8.1 mo) (Figure 1A and B).

Subgroup analysis

WHO PS was the only variable that was significantly associated with TTP and OS (Tables 2 and 3). Median TTP was 4.2 mo (95% CI: 3.2-7.0 mo) in PS 0 patients, 3.0 mo (95% CI: 1.8-4.1 mo) in PS 1 patients and 0.7 mo (95% CI: 0.5-1.5 mo) in PS 2 patients (Figure 2A). Median OS after the beginning of FOLFIRI was 8.2 mo (95% CI: 6.7-11.0 mo) in PS 0 patients, 5.4 mo (95% CI: 3.0-16.1 mo) in PS 1 patients and 2.5 mo (95% CI: 0.7-3.1 mo) in PS 2 patients (Figure 2B). PS 2 was significantly associated with a poor TTP [hazard ratio (HR) = 16.036, $P < 0.0001$] and OS (HR = 4.003, $P = 0.004$) in univariate analysis and in multivariate analysis also. No significant association was found between other variables and survival, except for the number of previous lines with TTP in multivariate analysis (Tables 2 and 3).

Dose adaptation and safety

Dose adaptation was required in 36 patients (57.1%).

Table 2 Results of univariate and multivariate analysis for time to progression with FOLFIRI

	Progression	Univariate analysis			Multivariate analysis		
	Yes/no (57/1)	HR	95% CI	*P* value	HR	95% CI	*P* value
Primary tumor location[1]				0.687			
Body or tail	18/0	1					
Head	28/1	0.883	(0.483-1.615)				
Stage at the diagnosis				0.666			0.652
Resectable	22/0	1			1		
Locally advanced	15/1	1.235	(0.631-2.418)		1.394	(0.662-2.939)	
Metastatic	20/0	0.905	(0.490-1.674)		1.299	(0.626-2.695)	
Previous surgery				0.659			
No	37/1	1					
Yes	20/0	0.882	(0.506-1.539)				
Previous radiotherapy				0.411			0.168
No	44/0	1			1		
Yes	13/1	1.305	(0.692-2.459)		1.770	(0.786-3.987)	
Number of previous chemotherapy lines				0.284			0.026
1	16/0	1			1		
2	36/1	0.649	(0.350-1.204)	0.170	0.385	(0.193-0.770)	
3	5/0	1.128	(0.411-3.096)	0.814	0.554	(0.177-1.735)	
Liver metastases				0.531			0.564
No	9/0	1			1		
Yes	48/1	0.793	(0.383-1.640)		1.298	(0.535-3.151)	
WHO performans status				< 0.0001			< 0.0001
0	24/0	1			1		
1	22/1	1.325	(0.731-2.399)		1.431	(0.745-2.748)	
2	11/0	16.036	(5.926-43.394)		29.255	(9.278-92.248)	
Type of FOLFIRI regimen				0.124			
FOLFIRI-1	50/0	1					
FOLFIRI-3	7/1	0.503	(0.210-1.207)				

HR: Hazard ratio; WHO: World Health Organization. [1]Data available in 47 out of 58 cases.

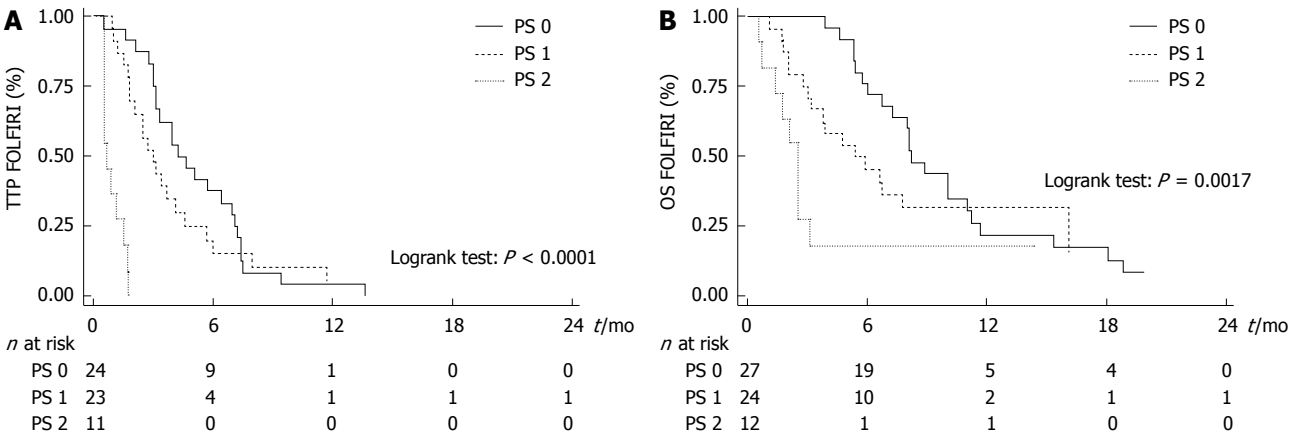

Figure 2 Subgroup analysis according to the World Health Organization performance status. A: Time to progression (TTP) according to World Health Organization (WHO) performance status (PS); B: Overall survival (OS) according to WHO PS.

The initial dose was reduced in 20 patients (31.7%) (19 patients who received the FOLFIRI-1 regimen and one patient who received the FOLFIRI-3 regimen) for the following reasons: cholestasis (n = 11), PS 2 (n = 8) or age > 75 years (n = 2), pre-existent diarrhea (n = 2) or mucositis (n = 1), and an episode of grades 3-4 hematological toxicity during previous chemotherapy (n = 1). A subsequent reduction was proposed in 19 patients (30.2%) (18 patients who received the FOLFIRI-1 regimen and one patient who received the FOLFIRI-3 regimen). Fifteen (23.8%) of these patients had grade 3-4 toxicities, mainly hematological (n = 11, 17.5%) and/or digestive with diarrhea and/or mucositis (n = 4, 6.3%). Febrile neutropenia occurred in 3 patients (4.8%). There were no related deaths.

DISCUSSION

We have evaluated the efficacy and safety of the FOLFIRI regimen after the failure of both gemcitabine and platinum salts in 63 patients with metastatic PAC treated in two centers. Tumor control was obtained in 39.7% of

Table 3 Results of univariate and multivariate analysis for overall survival with FOLFIRI

	Death	Univariate analysis			Multivariate analysis		
	Yes/no (49/14)	HR	95% CI	*P* value	HR	95% CI	*P* value
Primary tumor location[1]				0.306			
Body or tail	16/2	1					
Head	25/7	0.717	(0.380-1.355)				
Stage at the diagnosis				0.754			0.644
Resectable	20/4	1			1		
Locally advanced	13/6	1.012	(0.497-2.058)		0.790	(0.346-1.806)	
Metastatic	16/4	1.271	(0.643-2.515)		1.205	(0.577-2.514)	
Previous surgery				0.203			
No	31/9	1					
Yes	18/5	0.676	(0.370-1.236)				
Previous radiotherapy				0.916			0.935
No	38/9	1			1		
Yes	11/5	0.964	(0.491-1.894)		0.965	(0.412-2.260)	
Number of previous chemotherapy lines				0.102			0.171
1	14/5	1			1		
2	30/9	1.234	(0.649-2.346)		1.197	(0.564-2.538)	
3	5/0	3.188	(1.090-9.326)		2.945	(0.930-9.324)	
Liver metastases				0.599			0.957
No	9/3	1			1		
Yes	40/11	1.217	(0.586-2.528)		0.978	(0.444-2.159)	
WHO performance status				0.004			0.004
0	22/5	1			1		
1	18/6	1.360	(0.719-2.573)		1.284	(0.650-2.535)	
2	9/3	4.003	(1.770-9.056)		4.702	(1.883-11.741)	
Type of FOLFIRI regimen				0.856			
FOLFIRI-1	42/13	1					
FOLFIRI-3	7/1	1.083	(0.458-2.562)				

HR: Hazard ratio; WHO: World Health Organization. [1]Data available in 50 out of 63 cases.

patients. The median TTP was 3.0 mo and the median OS after the beginning of FOLFIRI was 6.6 mo. Toxicity was frequent with the FOLFIRI regimen (grade 3-4 toxicities in 23.8% of patients, mainly hematological and digestive) but manageable as only one patient had to stop treatment. In the subgroup analysis, the WHO PS was the only variable that was significantly associated with TTP (HR = 16.036, $P < 0.0001$) and OS (HR = 4.003, $P = 0.004$). Patients with WHO PS 2 may not benefit from this regimen.

Irinotecan-based chemotherapies have previously been tested in advanced PAC. Irinotecan was tested as a single agent in the first line setting in three phase Ⅱ trials with interesting results, showing an ORR of 9%-27% and a median OS of 5.2-7.3 mo[14-16]. However, two phase Ⅲ trials that tested irinotecan combined with gemcitabine as first-line chemotherapy did not show a significant benefit in TTP (2.8-3.5 mo) and OS (6.4-6.6 mo), despite a higher response rate than with standard gemcitabine (ORR: 15%-16%)[24,25]. A randomized phase Ⅱ study confirmed that the antitumoral activity of the combination of gemcitabine and irinotecan is similar to a regimen of fixed dose gemcitabine, gemcitabine/cisplatin or gemcitabine/docetaxel[26]. Thus, the gemcitabine/irinotecan combination does not seem to be synergistic. In contrast, the efficacy of the combination of irinotecan and 5-FU has been shown to be interesting with acceptable toxicity (Table 4)[20,27-29]. This is supported by *in vitro* and *in vivo* data showing synergy between these two drugs[17-19]. These regimens have not yet been tested in a phase Ⅲ trial compared with gemcitabine. Recently, a phase Ⅲ trial comparing the FOLFIRINOX regimen (folinic acid/5-FU, irinotecan and oxaliplatin combination) to gemcitabine as first-line treatment for metastatic PAC showed a significant improvement in survival with the FOLFIRINOX regimen with a median PFS and OS of 6.8 mo and 11.1 mo, respectively[7]. Toxicity was significant (grade 3-4 in 54% the patients) but manageable, and no toxic death occurred. Patients included in this study were in good condition (WHO PS 0-1). In addition, an absence of cholestasis was required for inclusion which probably explains the unusually high rate of body/tail tumor localization.

Irinotecan as a single agent has been shown to be a well-tolerated but marginally effective regimen in gemcitabine-pretreated patients. ORR was less than 10% and median OS did not exceed 4-6.6 mo[30,31]. The results with irinotecan-based combination regimens in gemcitabine-resistant advanced PAC were conflicting (Table 4)[32-37]. Data on irinotecan and 5-FU combination regimens in this setting are scarce. A randomized phase Ⅱ study evaluated modified FOLFIRI-3 *vs* modified FOLFOX (folinic acid/5-FU and oxaliplatin combination) in patients with gemcitabine-resistant advanced PAC. Efficacy was comparable with both regimens with a 6-mo OS rate of 27% (95% CI: 13%-46%) and 30% (95% CI: 15%-49%), respectively[21]. An Italian group reported a retrospective series of 40 patients with gemcitabine-

Table 4 Phase Ⅰ-Ⅱ studies of regimens as first and second lines chemotherapy in advanced pancreatic cancer

Regimen	Number of patients	TCR/ORR (%/%)	TTP or PFS (mo)	OS (mo)
Phase Ⅰ-Ⅱ studies[1]				
Irinotecan/gemcitabine/5-FU[27]	30	43/7	3.4	8.3
G-FLIP (Irinotecan/gemcitabine/5-FU/cisplatin)[28]	31	68/26	6.1	8.1
FOLFIRI-3 (Irinotecan/5-FU)[20]	40	65/38	5.6	12.1
Irinotecan/S1[29]	16	75/44	4.9	11.3
Phase Ⅱ studies[2]				
Irinotecan/raltitrexed[32]	19	53/16	4.0	6.5
IROX (Irinotecan/oxaliplatin)[33]	30	33/10	4.1	5.9
IROX (Irinotecan/oxaliplatin)[34]	14	50/21	1.4	4.1
G-FLIP (Irinotecan/gemcitabine/5-FU/cisplatin)[35]	34	44/24	3.9	10.3
Irinotecan/docetaxel[36]	14	21/0	1.2	4.4
MDI (Irinotecan/mitomycin/docetaxel)[37]	15	20/0	1.7	6.1

[1]Phases Ⅰ-Ⅱ studies of irinotecan and 5-fluorouracil (5-FU)-based regimens as first line chemotherapy; [2]Phase Ⅱ studies of irinotecan-based regimens as second line chemotherapy. TCR: Tumor control rate; ORR: Overall response rate; TTP: Time to progression; PFS: Progression free survival; OS: Overall survival.

resistant locally advanced or metastatic PAC treated with the standard FOLFIRI-1 regimen[22]. As in our series, most patients were PS 0-1 (82.5%); 17.5% of patients had locally advanced PAC, while all patients in our series were metastatic. The efficacy was quite similar to our series: TCR: 50%, ORR: 15%, median TTP: 3.7 mo and median OS: 6 mo. In contrast, toxicity was higher, with 27% and 32% of grades 3-4 hematological and digestive toxicities respectively. Toxicity was more frequent in PS 2 patients (71%) than in PS 0-1 patients (45%). The difference in the incidence of severe toxicity between the two series was not due to a difference in the proportion of PS 2 patients (17.5% *vs* 19%). One explanation might be that the initial dose was frequently adapted in our series (31.7%), particularly in PS 2 patients (8/12, 66.7%). In contrast, there was no tumor control at 6 mo in a study using a combination of irinotecan and fluoropyrimidine (mostly capecitabine) in 34 patients, most of whom where PS 0-1, and only 6% of patients were alive 1 year after the beginning of this chemotherapy regimen[38]. Most patients (97%) had been pretreated with capecitabine. This suggests that the different dose intensity and administration schedule for fluoropyrimidine, as in the XELIRI regimen, and the synergy of capecitabine and irinotecan could not overcome possible acquired resistance to this drug.

Our current study is the largest retrospective series on chemotherapy with a combination of irinotecan and 5-FU in metastatic PAC after the failure of gemcitabine and platinum salts. It was a bi-center study with a homogenous population (metastatic, not locally advanced, PAC) and selection bias was reduced by the systematic treatment policy in both centers. However, patients were heterogeneous regarding the number of previous lines of chemotherapy or the type of FOLFIRI regimen. We could not compare the efficacy of the two FOLFIRI regimens due to the unequal distribution of patients between the two groups, with only 8 patients receiving the FOLFIRI-3 regimen. Moreover, this study included selected patients treated in high volume centers with teams that were experienced in the management of pancreatic tumors and their complications. Endoscopic procedures for the treatment of jaundice, a classic exclusion criteria for irinotecan (40%-60% of PAC), were easily accessible. Another possible bias was the high rate of previous surgery (32.9%). The natural history of operated patients might be more favorable than that of patients with unresectable PAC at diagnosis. The toxicity of the FOLIFIRI regimen was manageable, although dose adaptation was required in more than half of patients (57.1%). Only patients who are PS 0-1 seem to benefit from this regimen.

In conclusion, the FOLFIRI regimen is a valuable option in patients with metastatic PAC after failure of gemcitabine and platinum salts but should be considered for patients in good condition (WHO PS 0-1). Further studies are needed to determine whether FOLFIRI is a valuable option as first line therapy in advanced PAC.

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COMMENTS

Background

Pancreatic adenocarcinoma (PAC) is the fourth leading cause of cancer death in the Western countries. The overall prognosis of metastatic PAC remains poor with a 5-year survival rate of less than 5%. Gemcitabine is the reference first-line regimen for metastatic PAC treatment. About half of patients with metastatic PAC whose disease progresses under gemcitabine are eligible for subsequent line(s) of chemotherapy and there is no standard regimen in that setting. Preclinical and clinical studies have suggested that the combination of 5-fluorouracil (5-FU) and irinotecan (FOLFIRI regimen) may be beneficial in PAC. The research aimed to evaluate the efficacy and safety of FOLFIRI regimen in patients with metastatic PAC after the failure of gemcitabine and platinum salts.

Research frontiers

Tumor response rate, toxicity of irinotecan-based regimen, time to progression and overall survival were determined for the FOLFIRI regimen.

Innovations and breakthroughs

This is a homogeneous study of consecutive metastatic PAC patients treated by two experienced teams in the management of patients with pancreatic cancer. The present paper suggests that whereas the combination of gemcitabine and irinotecan was not effective enough, that of 5-FU and irinotecan appears to be beneficial regarding both efficacy and tolerability. In addition, the study series

provides original data about the appropriate target population for the irinotecan-based regimen, particularly taking into account the PS status.

Applications

Recently, the FOLFIRINOX schema, combining 5-FU, irinotecan and oxaliplatin, was shown to be superior to gemcitabine in a first-line setting, with an overall survival of 11.1 mo (95% CI: 9-13.1 mo) *vs* 6.8 mo (95% CI: 5.5-7.6 mo, hazard ratio = 0.57) ($P < 0.0001$), respectively, in selected patients (performance status 0-1, absence of cholestasis). However, due to the hematological toxicity of this combination, many patients are not eligible for first-line therapy. The sequence FOLFOX then FOLFIRI (or the reverse) may be an alternative and should be considered as being better tolerated.

Terminology

For treatment purposes, pancreatic tumors are generally classified as resectable, locally advanced, or metastatic. A locally advanced pancreatic cancer is a tumor involving the arterial axis (celiac trunk, mesenteric artery) and thus is not resectable despite no detectable metastases. This form of cancer should be distinguished from metastatic tumors as the prognosis is different (slightly better, and some patients can have surgical treatment in case of a good tumor response after chemotherapy) and separate analyses are needed. Thus, locally advanced PAC patients were excluded from the study.

Peer review

This is an interesting study in which authors evaluate the efficacy and safety of this regimen in patients with metastatic PAC after the failure of gemcitabine and platinum salts. The results are convincing and suggest that FOLFIRI regimen had an acceptable toxicity and an interesting efficacy in our study, limited to patients in good condition.

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BRIEF ARTICLE

Efficacy of a therapeutic strategy for eradication of *Helicobacter pylori* infection

Giuliana Sereni, Francesco Azzolini, Lorenzo Camellini, Debora Formisano, Francesco Decembrino, Veronica Iori, Cristiana Tioli, Maurizio Cavina, Francesco Di Mario, Giuliano Bedogni, Romano Sassatelli

Giuliana Sereni, Francesco Azzolini, Lorenzo Camellini, Francesco Decembrino, Veronica Iori, Cristiana Tioli, Maurizio Cavina, Giuliano Bedogni, Romano Sassatelli, Unit of Gastroenterology and Digestive Endoscopy, Arcispedale Santa Maria Nuova, Istituto di Ricovero e Cura a Carattere Scientifico, 42123 Reggio Emilia, Italy
Debora Formisano, Statistics and Clinical Epidemiology Unit, Arcispedale Santa Maria Nuova, Istituto di Ricovero e Cura a Carattere Scientifico, 42123 Reggio Emilia, Italy
Francesco Di Mario, Unit of Gastroenterology, Ospedale S. Maria di Ca' Foncello, 31100 Treviso, Italy
Author contributions: Sereni G, Azzolini F, Camellini L, Decembrino F, Iori V, Tioli C, Cavina M and Sassatelli R treated and included the patients in the study and had access to the data; Azzolini F generated a computer-based database of all patients; Sereni G planned the study, organized and analyzed the data, discussed with all the authors the statistical analysis report and wrote the manuscript; Formisano D contributed to the statistical analysis of the data; Camellini L, Sassatelli R, Bedogni G and Di Mario F participated in the editing of the final report.
Correspondence to: Giuliana Sereni, MD, PhD, Unit of Gastroenterology and Digestive Endoscopy, Arcispedale Santa Maria Nuova, Istituto di Ricovero e Cura a Carattere Scientifico, Viale Risorgimento 80, 42123 Reggio Emilia, Italy. giuliana.sereni@asmn.re.it
Telephone: +39-522-296947 Fax: +39-522-295941
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Abstract

AIM: To determine the efficacy of our therapeutic strategy for *Helicobacter pylori* (*H. pylori*) eradication and to identify predictive factors for successful eradication.

METHODS: From April 2006 to June 2010, we retrospectively assessed 2428 consecutive patients (1025 men, 1403 women; mean age 55 years, age range 18-92 years) with gastric histology positive for *H. pylori* infection referred to our unit for 13-C urea breath test (UBT), after first-line therapy with proton pump inhibitor (PPI) *b.i.d.* + amoxicillin 1 g *b.i.d.* + clarithromycin 500 mg *b.i.d.* for 7 d. Patients who were still positive to UBT were recommended a second-line therapy (PPI *b.i.d.* + amoxicillin 1 g *b.i.d.* + tinidazole 500 mg *b.i.d.* for 14 d). Third choice treatment was empirical with PPI *b.i.d.* + amoxicillin 1 g *b.i.d.* + levofloxacin 250 mg *b.i.d.* for 14 d.

RESULTS: Out of 614 patients, still *H. pylori*-positive after first-line therapy, only 326 and 19 patients respectively rechecked their *H. pylori* status by UBT after the suggested second and third-line regimens. "Per protocol" eradication rates for first, second and third-line therapy were 74.7% (95% CI: 72.7%-76.4%), 85.3% (95% CI: 81.1%-89.1%) and 89.5% (95% CI: 74.9%-103%) respectively. The overall percentage of patients with *H. pylori* eradicated after two treatments was 97.8% (95% CI: 97.1%-98.4%), *vs* 99.9% (95% CI: 99.8%-100%) after three treatments. The study found that eradication therapy was most effective in patients with ulcer disease ($P < 0.05$, $P = 0.028$), especially in those with duodenal ulcer. Smoking habits did not significantly affect the eradication rate.

CONCLUSION: First-line therapy with amoxicillin and clarithromycin produces an *H. pylori* eradication rate comparable or superior to other studies and second-line treatment can still be triple therapy with amoxicillin and tinidazole.

Key words: *Helicobacter pylori*; Eradication treatment; Rescue therapy; Eradication rate; Triple therapy; First-line therapy; Second-line therapy

Peer reviewers: Dr. David J McGee, Microbiology and Immunology, Louisiana State University Health Sciences Center-Shreveport, 1501 Kings Highway, Shreveport, LA 71130, United States; Dr. Tamara Vorobjova, Department of Immunology,

University of Tartu, Ravila 19, 50411 Tartu, Estonia

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INTRODUCTION

Helicobacter pylori (*H. pylori*) is a bacterium that colonizes the stomach of about half the world's population and is considered the main cause of peptic ulcer[1,2] and mucosa-associated lymphoid tissue lymphoma[3,4]; furthermore it is an important risk factor for gastric adenocarcinoma[5,6] and also an associated factor for many gastric and non-gastric diseases[7]. *H. pylori* eradication is strongly recommended[8,9] in many clinical situations[10]. Different treatments have been proposed as effective[11,12] and meta-analyses are available comparing their results[13-15]. Prevalence of antibiotic resistance was found to be an important factor in the choice of the preferred regimen[16-19].

In particular, clarithromycin resistance is the strongest predictor of treatment failure[20,21]: in fact the eradication rate decreases below the recommended 80% threshold, when the prevalence of clarithromycin resistance reaches 15%-20%. On the other hand *in vitro* resistance to metronidazole may not accurately reflect resistance *in vivo*, and does not significantly affect the eradication rate[22]. In Northern Italy primary resistance to clarithromycin and metronidazole is reported to be 16.6% and 25.1% respectively[23].

The American College of Gastroenterology guidelines recommend first-line eradication with triple therapy consisting of a proton pump inhibitor (PPI), amoxicillin and clarithromycin. The European (Maastricht Ⅲ Consensus) guidelines recommend first-line eradication with triple therapy on account of clarithromycin/metronidazole resistance. According to both guidelines, second-line therapy should be a bismuth-based quadruple treatment while third-line treatment should be based on antimicrobial susceptibility testing[24].

Our Trust policy is to suggest an antibiotic regimen to outpatients referred to our unit for 13-C urea breath test (UBT), or esophagogastroduodenoscopy (EGD) with assessment of the *H. pylori* status and found to be *H. pylori*-positive. We do not usually prescribe a bismuth-based second-line therapy and we do not base the choice of the third-line therapy on antimicrobial susceptibility testing. The aim of the present study is to determine the efficacy of this strategy for *H. pylori* eradication.

MATERIALS AND METHODS

Setting

Our UBT outpatient clinic is the only existing one in the Health District of Reggio Emilia, serving about 500 000 people in North Italy. EGD is performed in six different public hospitals and in several private practices.

Patients

Our policy was to suggest an eradication therapy to the general practitioners of outpatients found to be *H. pylori*-positive after EGD or 13-C UBT performed at our Unit. Before examination (EGD or UBT), a brief medical history was obtained, including reason for investigation, allergy, past and ongoing treatments, and smoking habits. In patients undergoing EGD, if *H. pylori* status assessment was indicated, it was obtained by histology, according to the Sydney classification.

UBT was performed using an isotope ratio mass spectrometer (Breathmat, Bremen). A delta value > 3.5 over baseline was considered a positive result. All patients *H. pylori*-positive after EGD or UBT received a letter for their general practitioner reporting the result of the test, the recommended antibiotic regimen and the suggestion to further submit the patient to our UBT outpatient clinic to confirm *H. pylori* eradication, at least 8 wk after the end of treatment. Standard first-, second- and third-line antibiotic regimens are shown in Table 1. Alternative antibiotic regimens were suggested to patients with known allergy to a particular antibiotic. Each patient also received a form, where she/he was instructed to mark consumption of the drugs and to record any suspected adverse effect. Compliance with the proposed treatment and adverse effects were checked, on the occasion of the resubmission of the patient. Nevertheless no attempt was made to contact patients, who did not return to our UBT outpatient clinic and no information about them was actively sought. Data about patients and treatments were prospectively collected in a purpose-built database.

In the present observational retrospective study, we included 2428 outpatients (Table 2 shows their characteristics), who underwent UBT from April 2006 to June 2010, after positive histological assessment of *H. pylori* status and after first-line eradication therapy with PPI *b.i.d.* + amoxicillin 1 g *b.i.d.* + clarithromycin 500 mg *b.i.d.* for 7 d.

Ethics and endpoints

The study was conducted according to the declaration of Helsinki. All patients consented to UBT. Although patients were informed about the purpose and potential side effects of the suggested treatments, a formal consensus about therapy was not obtained, because the therapy was always finally prescribed by the general practitioner after further evaluation and discussion with the patient. Furthermore none of the medications were prescribed as part of a clinical trial and therefore there was no need for study approval by the ethics committee at our hospital. The primary endpoints of the study were eradication rates of the suggested antibiotic regimens, as calculated per protocol in patients, who were referred to our UBT outpatient clinic after each line of treatment. Secondary endpoints were: (1) percentage of patients, still *H. pylori*-positive after first-line therapy, who achieved

Table 1 Antibiotic regimens suggested by our unit and eradication rates per protocol

	Patients	*H. pylori* eradicated and eradication rate per protocol (95% CI)	Patients treated and cumulative effect (95% CI)
First-line: PPI *b.i.d.* + amoxicillin 1 g *b.i.d.* + clarithromycin 500 mg *b.i.d.* for 7 d	2428	1814 74.7 (72.7-76.4)	
Second-line: PPI *b.i.d.* + amoxicillin 1 g *b.i.d.* + tinidazole 500 mg *b.i.d.* for 14 d	326	278 85.3 (81.1-89.1)	2140 97.8 (97.1-98.4)
Third-line: PPI *b.i.d.* + amoxicillin 1 g *b.i.d.* + levofloxacin 250 mg *b.i.d.* for 14 d	19	17 89.5 (74.9-103)	2111 99.9 (99.8-100)

PPI: Proton pump inhibitor; b.i.d.: Twice daily; *H. pylori*: *Helicobacter pylori*. Standard dosages for PPIs are as follows: lansoprazole 30 mg, omeprazole 20 mg, pantoprazole 40 mg, rabeprazole 20 mg, esomeprazole 20 mg.

Table 2 Characteristics of patients who underwent urea breath test after treatment (%)

Patients characteristics	UBT after first-line therapy (2428 patients)	UBT after second-line therapy (326 patients)	UBT after third-line therapy (19 patients)
Women	1403 (58)	202 (62)	13 (68)
Men	1025 (42)	124 (38)	6 (32)
Mean age (range), yr	55 (18-92)	54 (21-86)	53 (27-80)
Peptic ulcer disease	202 (8.2)[1]	23 (7.1)	0
Previous gastric surgery and/or neoplasm	6 (0.2)[2]	3 (0.9)	0
Smokers	424/1082 (43.8)	59/287 (20.6)	3/17 (7.6)

[1]172 and 23 patients were found to be affected respectively by duodenal and gastric ulcer. 7 patients were affected by both gastric and duodenal ulcer; [2]1 patient was affected by low-grade mucosa-associated lymphoid tissue. Among the patients with a history of previous gastric surgery, 1 patient had undergone surgery for cancer, the others for peptic ulcer disease. UBT: Urea breath test.

H. pylori eradication, proved by UBT, at the end of study; and (2) factors predictive of successful eradication.

Statistical analysis

The data was analysed with SPSS v.18.0 (Chicago, Illinois United States). The significance level is 0.05. The data is summarized by the primary measures of central tendency and dispersion, as well as with frequencies and percentages with 95% CI. Pearson's Chi square and Fisher's exact test were used to compare frequencies, where appropriate.

RESULTS

The study flow chart is shown in Figure 1. Six hundred and fourteen patients (25.3%) were found to be *H. pylori*-positive to UBT, after the first-line antibiotic regimen.

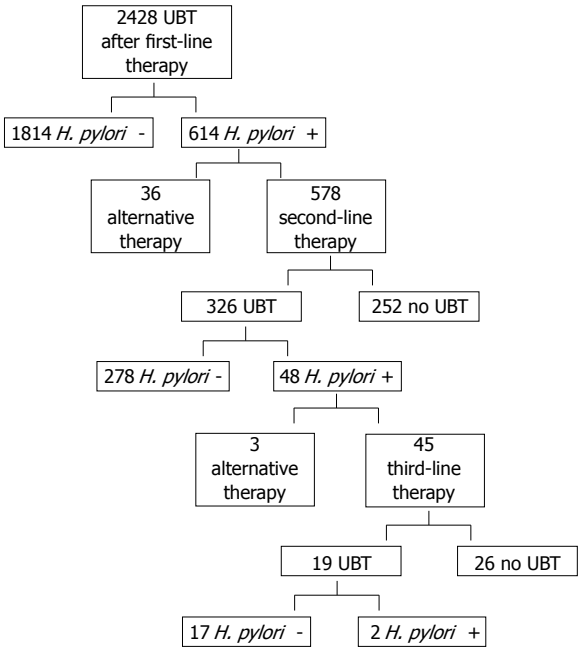

Figure 1 Flow chart of the study. UBT: Urea breath test; *H. pylori*: *Helicobacter pylori*.

Out of these patients, 326 (53.1%) were referred again to our unit to check eradication after second-line standard therapy. Out of the 48 still *H. pylori*-positive after second-line therapy, only 19 underwent UBT (39.6%).

An antibiotic regimen alternative to the second or third-line standard therapy was suggested by us, or by their general practitioner, to 40 patients (6.5%). Among the patients, who were referred to us for UBT, a small number reported a non-complete compliance with the received standard antibiotic regimen, because of side effects: this was the case in 13 patients (0.5%, 11 tested *H. pylori*-negative and 2 positive) during first-line therapy and in 9 patients (2.7%, all eradicated) during second-line therapy. Three out of 21 reported a previously unknown allergy to penicillin; all other side effects were minor (diarrhea, headache, metallic taste).

Eradication rates of the antibiotic regimen, as calculated in patients who subsequently checked *H. pylori* status by UBT, are reported in Table 1. The two patients who were still positive after the third treatment were advised to take bismuth-based quadruple therapy for 14 d and *H. pylori* was finally eradicated.

As shown in Table 3, peptic ulcer disease, but not smoking habits, was found to be predictive of successful eradication during first-line therapy. Stratifying patients according to the number of cigarettes smoked per day (data not shown), the eradication rate decreased proportionally to the increase in the number of cigarettes, though statistical significance was only borderline ($P = 0.056$).

Table 4 compares that characteristics of the patients who rechecked (297, 48.4%) or not (278) their *H. pylori* status, until the achievement of an UBT-proved *H. pylori*-negative status, or after the completion of third-line therapy, whichever came first.

Table 3 Factors investigated as potentially predictive of successful eradication (%)

Risk factor	Eradication rate	
	First-line antibiotic regimen	Second-line antibiotic regimen
Women	1029/1403 (73.3)	172/202 (85.1)
Men	785/1025 (76.6)	106/124 (85.5)
Peptic ulcer disease	167/206 (81.1)[1]	22/24 (91.7)
Non-ulcer disease	1647/2222 (74.1)[1]	54/71 (76.1)
Smokers	302/424 (71.2)	50/57 (94.7)
Non-smokers	1346/1 778 (75.7)	192/230 (83.5)

[1]Difference of frequencies: 6.9% (95% CI: 1%-12.8%, $P < 0.05$). If patients affected by duodenal ulcers is compared with patients with non-ulcer disease, the difference is 8.3% (95% CI: 2.3%-14.4%, $P < 0.05$).

Table 4 Characteristics of patients who rechecked or not their *Helicobacter pylori* status (%)

Patients characteristics	Rechecked their *H. pylori* status[1] (297 patients)	No recheck of their *H. pylori* status (278 patients)
Women/men	185 (62)/112 (38)	170 (61)/108 (39)
Mean age (range), yr	55 (21-86)	50 (20-92)
Peptic ulcer disease	21 (7)	17 (6)
Previous gastric surgery and/or neoplasm	2 (0.6)	1 (0.3)
Smokers	53 (20)	69 (27)

[1]Patient who rechecked their *Helicobacter pylori* (*H. pylori*) status, until the achievement of an urea breath test-proved *H. pylori*-status, or after the completion of the third-line therapy, whichever came first. Patients treated with alternative second and third-line antibiotic regimens are excluded.

DISCUSSION

In our study, patients were treated with first-line PPI-amoxicillin-clarithromycin therapy for 7 d only. A recent meta-analysis showed a minimal advantage of longer treatment[25].

All the different PPIs (omeprazole, lansoprazole, pantoprazole, rabeprazole and esomeprazole) are shown to be equally effective in combination with antibiotic therapy[26] and higher H2 receptor antagonists (ranitidine)[27]: therefore we left the choice of PPI to the general practitioner. We also advised general practitioners to prescribe the double dose of PPI, because it has proved more effective in a recent meta-analysis[28].

The eradication rate after first therapy was 74.7%. This rate is comparable to the 76% achieved by the same regimen (for 10 d of treatment) in a recent study from Greece[29] and it is higher than the 56% found in a study with 14 d of PPI-clarithromycin-metronidazole[30,31].

The European and American guidelines recommend a bismuth-based quadruple treatment as second-line therapy; according to Maastricht guidelines, a PPI-amoxicillin-metronidazole therapy could be used if bismuth is not available.

In Italy bismuth salts are often available, but we preferred to recommend PPI-amoxicillin-tinidazole as second-line therapy, because the bismuth-based quadruple regimen is associated with a relatively high incidence of side effects and a worse compliance than the triple regimen; although a recent meta-analysis shows no statistically significant differences[32]. The PPI-amoxicillin-metronidazole regimen was reported to be effective as second-line therapy to achieve eradication rates of 89% and 64% for metronidazole susceptible and resistant strains, respectively[33]. In our study, this second-line regimen obtained an eradication rate of 85.3%. These results are superior to data reported by others after unsuccessful first-line treatment for the same regimen, for the bismuth-based quadruple combination[34] and also for a levofloxacin-based therapy[35].

The Consensus Conference recommended third-line rescue treatment based on antimicrobial susceptibility testing. We preferred not to perform susceptibility testing, choosing an empirical triple therapy with levofloxacin.

Two meta-analyses have suggested that levofloxacin-based rescue regimen is more effective and safer than quadruple therapy[35,36]. Levofloxacin resistance develops rapidly and it is one of the most effective antibiotics in the treatment of respiratory tract infections. For these reasons, we reserved its use for third-line therapy. In our study, levofloxacin-based third-line therapy achieved an eradication rate of 89.5%. Although we observed these excellent results in a small group of patients, our findings are consistent with other experiences[29,36] and support the use of a levofloxacin-based regimen as empirical third-line therapy of *H. pylori* infection.

The overall eradication rate after two and three treatments, respectively, is proved to be 97.8% and 99.9%. These results confirm the data already published in literature (Table 5).

During first-line treatment, the efficacy of therapy in patients with peptic ulcer disease was higher (81.1%) than in patients with non-ulcer disease (74.1%). This finding is in agreement with others experiences[37-39] and could be explained by a larger number of antibiotic-resistant *H. pylori* strains in non-ulcer disease patients.

The study also assessed the influence of smoking on treatment: the eradication rate was moderately higher in non-smokers (75.7% *vs* 71.2%). Recent studies demonstrated that smoking affects the eradication rate[40,41]; in our larger report the trend is negative but not yet statistically significant.

It should be noted that our study is not a clinical trial, but a retrospective revision of our practice. This may explain its major limitation; that is the high number of patients (45.3%), who did not have their *H. pylori* status rechecked after failure of first-line therapy. We do not know whether these patients did not undergo any further treatment, or they stopped therapy because of adverse effects, or decided not to repeat UBT after completion of the suggested regimen. Nevertheless the regimens suggested in our studies are usually relatively well tolerated and we do not believe that the high percentage of our patients lost at follow up was due to adverse effects. In the setting of an open-access UBT facility,

Table 5 Studies reporting eradication rates and cumulative effect of different therapeutic strategies

	% Eradication rate (number of patients)				
	First-line	Second-line	Third-line	Cumulative effect (Ⅱ to Ⅲ line)	Antimicrobial susceptibility testing before third-line
Gasbarrini *et al*[41] 2000	86 (2413)	82 (329)	77 (39)	99	Yes
Beales[42] 2001	73 (469)	70-73 (66)	65 (20)	94-98	Yes
Qasim *et al*[40] 2005	77 (3280)	56 (270)	38 (28)		
Gisbert *et al*[32] 2008	70 (500)	74 (343)	76 (136)	99.5	No
Rokkas *et al*[28] 2009	76 (540)	73 (120)	70 (30)	98	No
Present study	75 (2428)[1,2]	85 (326)	89 (19)	97.8-99.9	No

[1]172 and 23 patients were found to be affected respectively by duodenal and gastric ulcer. 7 patients were affected by both gastric and duodenal ulcer; [2]1 patient was affected by low-grade mucosa-associated lymphoid tissue. Among the patients with a history of previous gastric surgery, 1 patient had undergone surgery for cancer, the others for peptic ulcer disease. UBT: Urea breath test.

Qasim *et al*[40] reported that only 25% of their patients *H. pylori*-positive after first-line therapy, were proved to have eradication of *H. pylori* after second-line therapy or have rechecked their *H. pylori* status by UBT after third-line therapy. A high compliance with repeated courses of therapy is reported in prospective clinical trials[29,41], but it is conceivable that it may be difficult to replicate these good results in common clinical practice. Data are lacking about strategies useful to reinforce compliance with *H. pylori* eradication therapy in this setting. It should be noted that a high percentage of our patients did not achieve a proved *H. pylori*-negative status, despite the fact that our policy avoided invasive testing (EGD) to obtain antimicrobial susceptibility testing and regimens requiring assumption of a lot of pills, such as bismuth-based quadruple therapy.

We believe that efforts to introduce new effective antibiotic regimens, should parallel not only the emergence of new resistance, but also the efforts to target appropriate antibiotic regimens to the majority of patients requiring *H. pylori* eradication therapy. Our policy to suggest to the general practitioner an antibiotic regimen for each patient found to be *H. pylori*-positive may be criticized and it could be not generally applicable. Nevertheless different models of collaboration between gastroenterologists and general practitioners should be tested to improve effectiveness of *H. pylori* infection therapy in current practice.

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We appreciate that Giuliana Sereni, MD, PhD, would like to polish language and provide language certificate letter.

COMMENTS

Background

Helicobacter pylori (*H. pylori*) is considered an important risk factor for many gastric and non-gastric diseases. Different treatments have been proposed as effective for *H. pylori* eradication and meta-analyses are available comparing their results. Prevalence of antibiotic resistance was found to be an important factor in the choice of the preferred regimen.

Research frontiers

The European (Maastricht Ⅲ Consensus) and American (American College of Gastroenterology) guidelines recommend a therapeutic strategy for *H. pylori* eradication with clarithromycin and metronidazole for 7-14 d or amoxicillin and clarithromycin for 10-14 d as first-line therapy. They also suggest bismuth-based quadruple second-line therapy and third-line therapy based on antimicrobial susceptibility tests.

Innovations and breakthroughs

In the present study, about 75% of patients were cured with first-line therapy (proton pump inhibitor + amoxicillin + clarithromycin), 97.8% after the second-line therapy (proton pump inhibitor + amoxicillin + tinidazole) and 99.9% after the third-line therapy (proton pump inhibitor + amoxicillin + levofloxacin). No statistical differences in eradication rates in smokers and non-smokers were found, even if there is a negative trend. Patients with ulcer disease had an eradication rate superior to that of patients with non-ulcer disease. This study was conducted in an open access setting and showed only about half of the patients still *H. pylori*-positive after first-line therapy were finally proved to have successful eradication of *H. pylori*.

Applications

The high eradication rates of this study suggest that a regimen simpler than quadruple drug therapy may be preferred as second-line therapy at least in some patients and in some regions; furthermore the antimicrobial susceptibility test (and the esophagogastroduodenoscopy involved) would not be necessary after two failed treatments. The fact that nearly half of our patients still *H. pylori*-positive after first-line therapy did not reach a urea breath test (UBT)-proved *H. pylori*-negative status, despite the use of effective antibiotic regimens, suggests that strategies should be devised and implemented to improve the access of patients to appropriate therapies and to strengthen the compliance of patients to these treatments.

Terminology

H. pylori is a bacterium that colonizes the stomach of about half the world's population and is considered the main cause of peptic ulcer and mucosa-associated lymphoid tissue lymphoma; furthermore it is an important risk factor for gastric adenocarcinoma and also an associated factor for many gastric and non-gastric diseases. *H. pylori* infection can be detected by esophagogastroduodenoscopy (a biopsy is taken during endoscopy and it is sent to the hospital laboratory to be examined for histology) or UBT. In the 13-C UBT, person fast for about 6 h. A baseline breath sample is collected (person blow into a test tube), then person drink a solution of Carbon-13-urea in water. A second breath sample is taken after 30 min and analyzed by a mass spectrometer. If *H. pylori* is present in the stomach the Carbon-13-urea will be broken down and Carbon-13 will appear in the breath.

Peer review

These authors conducted a retrospective epidemiology study on over 2428 patients who were positive for *H. pylori* looking to determine the efficacy of a therapeutic strategy for *H. pylori* eradication based on first-line therapy with amoxicillin and clarithromycin and second-line treatment with amoxicillin and tinidazole. The authors found that the eradication therapy was most effective in patients with ulcer disease, especially with duodenal ulcer during first-line therapy.

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BRIEF ARTICLE

Lack of CD44 variant 6 expression in rectal cancer invasive front associates with early recurrence

Suvi Tuulia Avoranta, Eija Annika Korkeila, Kari Juhani Syrjänen, Seppo Olavi Pyrhönen, Jari Toivo Tapio Sundström

Suvi Tuulia Avoranta, Eija Annika Korkeila, Kari Juhani Syrjänen, Seppo Olavi Pyrhönen, Department of Oncology and Radiotherapy, University of Turku and Turku University Hospital, FIN-20521 Turku, Finland
Jari Toivo Tapio Sundström, Department of Pathology, University of Turku and Turku University Hospital, FIN-20520 Turku, Finland
Author contributions: All authors designed the research; Avoranta ST and Korkeila EA collected the clinicopathological data; Avoranta ST and Sundström JTT analyzed the immunohistochemical staining; Avoranta ST and Syrjänen KJ performed the statistical work; Avoranta ST wrote the paper; Korkeila EA, Syrjänen KJ, Pyrhönen SO and Sundström JTT gave comments and suggested revisions.
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Correspondence to: Tuulia Avoranta, MD, Department of Oncology and Radiotherapy, University of Turku and Turku University Hospital, Hämeentie 11, FIN-20521 Turku, Finland. stavor@utu.fi
Telephone: +35-82-3131834 Fax: +35-82-3132809
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Abstract

AIM: To investigate the prognostic value of CD44 variant 6 (CD44v6), a membranous adhesion molecule, in rectal cancer.

METHODS: Altogether, 210 rectal cancer samples from 214 patients treated with short-course radiotherapy (RT, *n* = 90), long-course (chemo) RT (*n* = 53) or surgery alone (*n* = 71) were studied with immunohistochemistry for CD44v6. The extent and intensity of membranous and cytoplasmic CD44v6 staining, and the intratumoral membranous staining pattern, were analyzed.

RESULTS: Membranous CD44v6 expression was seen in 84% and cytoplasmic expression in 81% of the cases. In 59% of the tumors with membranous CD44v6 expression, the staining pattern in the invasive front was determined as "front-positive" and in 41% as "front-negative". The latter pattern was associated with narrower circumferential margin (P = 0.01), infiltrative growth pattern (P < 0.001), and shorter disease-free survival in univariate survival analysis (P = 0.022) when compared to the "front-positive" tumors.

CONCLUSION: The lack of membranous CD44v6 in the rectal cancer invasive front could be used as a method to identify patients at increased risk for recurrent disease.

Key words: CD44 variant 6; Rectal cancer; Invasive front; Disease-free survival; Disease-specific survival

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INTRODUCTION

In 2008, approximately 1.2 million new cases of colorectal cancer (CRC) were diagnosed globally[1]. Preoperative radiotherapy (RT) for rectal cancer has improved local control rates[2], but patients may still present with fairly differing clinical responses and prognoses[3]. To improve

disease predictability, strong expectations have been placed on tumor markers.

CD44 is a family of transmembrane glycoproteins serving as a major receptor for hyaluronate, an important component of the extracellular matrix (ECM)[4]. *CD44* has been suggested to act both as a tumor-suppressing cofactor and as a growth- and invasiveness-promoting molecule[5] through its participation in many important cellular processes, including adhesion, growth regulation, survival, differentiation and motility[6]. Of the several isoforms produced by alternative splicing of the CD44 gene[7], variant 6 (CD44v6) has been intensely studied in relation to CRC progression and outcome[8].

Induction in CD44v6 expression is suggested to represent an early event in colorectal carcinogenesis[9], and has in some studies been related to disease progression, metastatic potential[7,9,10], and poor disease outcome[11]. Instead, in some other studies, stronger CD44v6 expression has been reported in adenomas than in carcinomas, as well as in primary carcinomas compared to metastatic tumors[12], and has been shown to have favorable[8,13] or no[14] effect on CRC outcome. Furthermore, strong expression has been shown to indicate more favorable response to chemotherapy[15].

In most of the previous studies on CD44v6 expression in colorectal tumors, both colonic and rectal carcinomas have been included. There is, however, some evidence that proximal and distal colonic lesions differ in their expression of CD44v6[16]. In those few studies including solely rectal tumors[17-19], CD44v6 has not been considered in relation to RT. In the present study, we examined the expression of CD44v6 immunohistochemically in a cohort of 214 primary rectal carcinomas treated with or without preoperative (chemo)radiotherapy. Considering the fundamental role of the tumor invasive front in tumor-host interaction[20], as well as the discrepant data on the prognostic value of the extent of CD44v6 expression in CRC[8,11,14], intratumoral staining pattern of this protein was also systematically assessed. We hypothesized that this aspect could offer additional information of the significance of CD44v6 expression in rectal cancer.

MATERIALS AND METHODS

Patients and study material

The material of this study consisted of formalin-fixed, paraffin-embedded tissue samples from 214 patients operated upon for rectal cancer at Turku University Hospital between 2000 and 2009. Operative samples were retrieved from the archives of the Department of Pathology, Turku University Hospital. To ensure a biologically and therapeutically homogeneous study population, only tumors of the middle and lower rectum were included. Superficial tumors treated with excision only, as well as patients with distant metastases at the time of diagnosis, were excluded. The use of archival tissue material was approved by the National Supervisory Authority for Welfare and Health (permission No. Dnro 1709/32/300/02, May 13th 2002).

Tumor staging was done according to the tumor node metastasis classification of malignant tumors, 2002[21]. Selection of treatment was based on preoperative tumor staging which included computed tomography (CT) or magnetic resonance imaging of the rectum, CT of the abdomen, and X-ray or CT of the chest. According to the common clinical guidelines[22], patients were treated either with short-course preoperative RT (n = 90), long-course preoperative (chemo) RT (n = 53), or received no treatment before surgery (n = 71). Short-course RT consisted of five 5-Gy fractions during 1 wk, with surgery on the following week. Long-course RT was given in 1.8-Gy fractions to a total dose of 50.4 Gy over a 6-wk period, with (n = 44) or without (n = 9) concomitant chemotherapy, and operation was performed at 5-7 wk after RT. Chemotherapy regimens were either bolus 5-fluorouracil (n = 5) or capecitabine (n = 39). Anterior resection was performed in 118 cases (55%), and abdominoperineal resection in 92 cases (43%). In four cases (2%), some other technique, such as low Hartmann's procedure, was used. The presence of vascular invasion was assessed in 159 cases, in which cancer cells could be detected in 47 cases (30%), either in the extramural lymphatic or blood vessels. Postoperative adjuvant chemotherapy was given to patients with lymph node positive or high-risk lymph node negative tumors according to the standard clinical practice[22]. The median follow-up time was 45.5 mo. In 61 patients (29%), local or distant disease recurrence was seen. Of them, 39 (64%) were treated with chemotherapy with or without biological treatments. Among these 39 cases, progression-free survival (PFS) was retrospectively defined from the medical records, and the median PFS was 20.7 mo. The key demographic and clinical characteristics of the patients are summarized in Table 1.

Immunohistochemistry

Four tumors with T0 after long-course RT were not stained, and accordingly, the actual study material consisted of 210 samples. The most representative blocks were selected and cut into 5-μm sections. Antigen retrieval was performed by heating in a microwave oven in 10 mmol/L sodium citrate, pH 6.0 two times for 7 min. Endogenous peroxidase activity was blocked by incubating the slides in 0.3% hydrogen peroxide in Tris-buffered saline. The sections were subjected to immunohistochemical staining with monoclonal antibody for CD44v6 (concentration 1:1000; Bender MedSystems, Vienna, Austria) and monoclonal mouse anti-cytokeratin (Ck-Pan) (clone: AE1/AE3, concentration 1:50; Zymed Laboratories, South San Francisco, CA, United States) using EnVision + Dual Link System - HRP (Dako, Denmark).

Evaluation of immunohistochemistry for CD44v6

Immunohistochemistry (IHC) staining was individually evaluated by two observers (Avoranta ST and Sundström

Table 1 Clinical characteristics of the patients *n* (%)

Clinical characteristics	Short course RT	Long course RT	Control	P^1
Study population (n = 214)				
Male	57 (63)	34 (64)	32 (45)	0.03
Female	33 (37)	19 (36)	39 (55)	
Mean age (yr)	65.2	64.7	74.5	< 0.001
Preoperative T				
T1-2	28 (31)	0 (0)	22 (31)	
T3	54 (60)	2 (4)	12 (17)	< 0.001
[2]T4	1 (1)	50 (94)	3 (4)	
Tx	7 (8)	1 (2)	34 (48)	
Postoperative T				
T1	3 (3)	2 (4)	5 (7)	
T2	34 (38)	6 (11)	26 (37)	
T3	50 (56)	27 (51)	37 (52)	< 0.001
T4	3 (3)	14 (26)	3 (4)	
T0	0	4 (8)	0	
Postoperative N				
N0	53 (59)	36 (68)	41 (58)	
N1	26 (29)	14 (26)	15 (21)	0.33
N2	11 (12)	3 (6)	12 (17)	
Nx	0	0	3 (4)	
Grade				
G1	9 (10)	10 (19)	12 (17)	
G2	58 (64)	33 (62)	48 (68)	0.07
G3	21 (23)	3 (6)	11 (16)	
Gx	2 (2)	7 (13)	0	
[3]Crm (mm)				
0	3 (4)	11 (25)	4 (9)	
0 ⩽ crm ⩽ 2	9 (12)	7 (16)	7 (16)	0.009
> 2	65 (84)	26 (59)	32 (74)	
Disease specific outcome				
Alive without recurrence	64 (71)	25 (47)	39 (55)	
Alive with recurrence	7 (8)	7 (13)	4 (6)	0.07
Died of disease	11 (12)	12 (23)	18 (25)	
Died of other causes	8 (9)	9 (17)	10 (14)	

[1]Differences between the three treatment groups using Fisher's exact test; mean age with analysis of variance; [2]Includes the T3 tumors with threatened circumferential margin involvement; [3]Circumferential margin, data available in 164 cases. RT: Radiotherapy.

JTT) blinded to clinical data. Squamous basal cells of anal epithelium included in some of the cases were used as a positive control for CD44v6. Using 5×, 10× and 20 × objectives, the immunostaining of CD44v6 in every slide was assessed using the following parameters: (1) the extent of expression (membranous and cytoplasmic staining scored separately); (2) the intensity of expression (membranous and cytoplasmic staining scored separately); and (3) the intratumoral pattern of membranous CD44v6 expression. The extent of expression within the whole tumor area was analyzed using four categories: 1 for immunopositivity < 5%, 2 for 5%-20%, 3 for 21%-50% and 4 for > 50%. The intensity of expression was analyzed using four categories: 1 for negative staining; 2 for weak staining (a faint immunopositivity in the membrane or cytoplasm); 3 for moderate staining (a clear immunopositivity in the membrane or cytoplasm); and 4 for strong staining (a pronounced immunostaining in the membrane or cytoplasm equivalent to that of the basal cells of anal squamous epithelium). The intratumoral pattern of membranous CD44v6 expression was classified into three categories: 1 for immunopositivity mainly in the tumor invasive front; 2 for immunopositivity mainly in the tumor central areas; and 3 for heterogenous immunopositivity (no apparent difference in CD44v6 expression between the invasive front and central areas of tumor).

For statistical purposes, categories 1 and 2 were studied as one group and categories 3 and 4 as another group when analyzing the extent and intensity of expression. With regard to the intratumoral pattern of membranous staining, categories 1 and 3 were studied as one group ("front-positive"), and category 2 as in its own group ("front-negative").

Evaluation of tumor growth pattern

In order to evaluate tumor growth pattern, hematoxylin-eosin and Ck-Pan stainings were scrutinized in each operative sample. Using the Jass' classification of the tumor growth pattern, tumours were appointed as "expanding" when the invasive margin was pushing or reasonably well circumscribed, and "infiltrating" when the tumor invaded in a diffuse manner with widespread penetration into adjacent normal tissues[23].

Evaluation of tumor regression grade

Tumor regression grade (TRG) after long-course RT was defined by a pathologist (Sundström JTT) as poor, moderate or excellent according to the modified Dworak and Rödel scales, as described recently[24]. Briefly, poor TRG was defined as minimal or no tumor regression after (chemo) RT. In case of poor response, many tumor cells remained after treatment. In tumors with moderate response, there were only some tumor cells or tumor cell groups left in the primary tumor, lymph nodes or perirectal fat. In tumors with excellent response, few or no tumor cells could be detected.

Statistical analysis

Statistical analysis were run using IBM SPSS® Statistics 19.0.1 for Windows software. Frequency tables were analyzed using the χ^2 test, with the likelihood ratio (LR) or Fisher's exact test for categorical variables. 2 × 2 tables were used to calculate odds ratio and 95% CI using the exact method. Fisher's exact test, Spearman's correlation and LR were used to assess the significance of the correlation between individual variables in univariate analysis. Inter-observer reproducibility of the assessments was tested using weighted κ. It was calculated using the intraclass correlation coefficient (ICC) test, in parallel mode with a two-way random model, using consistency assumption and the average-measures option to interpret the ICC (95% CI). The ICC of assessments was very good with weighted κ ranging from 0.70 to 0.90.

Univariate survival analysis for disease-free survival (DFS) and disease-specific survival (DSS) was based on the Kaplan-Meier method where stratum-specific outcomes were compared using log-rank (Mantel-Cox) statistics. To adjust for the covariates, a Cox proportional

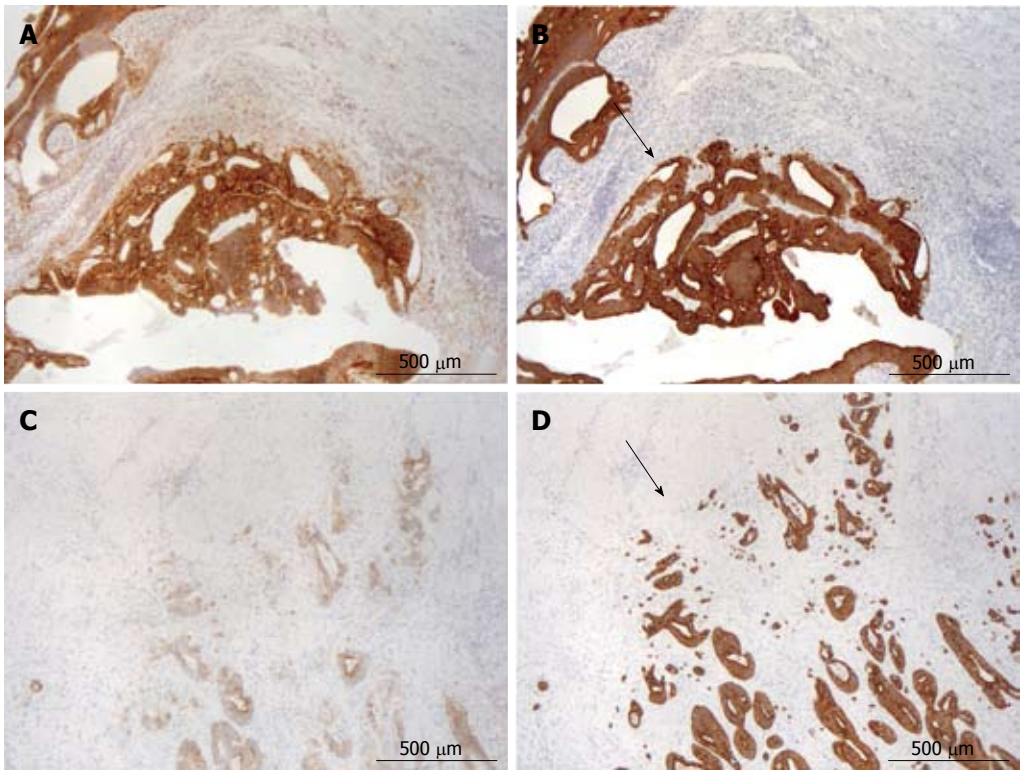

Figure 1 The immunohistochemistry for CD44 variant 6 and Pan-cytokeratin. A: The same tumor with "front-positive" membranous staining of CD44 variant 6 (CD44v6); B: Expanding growth pattern as shown with Pan-cytokeratin (Ck-Pan) staining; C: The same tumor with "front-negative" membranous staining of CD44v6; D: Infiltrating growth pattern as shown with Ck-Pan staining. Invasive front is indicated with arrows.

hazards regression model was used, covariates (as listed separately) being inserted using the enter mode. Of the variables significant in univariate analysis, two (circumferential margin, vascular invasion) were not included in the multivariate model because the data were incomplete. All statistical tests were two-sided and declared significant at a P value of < 0.05.

RESULTS

CD44v6 expression in tumors

Altogether, 177 out of 210 tumors (84%) showed membranous and 170 (81%) cytoplasmic CD44v6 expression. Both the extent and intensity of membranous and cytoplasmic staining were closely correlated ($r = 0.4$; $r = 0.31$, $P < 0.001$ for both). Of the 177 tumors with membranous CD44v6 positivity, 105 (59%) were "front-positive" (Figure 1A), usually showing an expanding growth pattern (Figure 1B), and 72 (41%) were "front-negative" (Figure 1C), usually showing an infiltrating growth pattern (Figure 1D).

Tumor growth pattern

In 206 tumors, a proper invasive front could be detected to evaluate tumor growth pattern. Altogether, 99 (48%) showed an expanding (Figure 1B) and 107 (52%) an infiltrating growth pattern (Figure 1D).

CD44v6 related to treatment groups and TRG

Only the extent of cytoplasmic staining was related to treatment group. Patients in the long-course RT group had less cytoplasmic CD44v6 expression in their tumors as compared to patients in the control group ($P = 0.002$). Within the long-course RT group, no differences in CD44v6 expression were seen according to concomitant chemotherapy. TRG after long-course RT, as analyzed also from the four tumors without IHC for CD44v6, was poor in 27 cases (51%), moderate in 14 cases (26%) and excellent in 12 cases (23%). No significant differences were seen in CD44v6 expression, as related to TRG.

CD44v6 related to clinicopathological variables

No significant differences were seen in the extent or intensity of CD44v6 expression concerning the variables listed in Table 1. With regard to the intratumoral pattern of membranous staining, "front-negative" was associated with narrower circumferential margin ($P = 0.01$), infiltrative tumor growth pattern ($P < 0.001$), and a greater risk for disease recurrence ($P = 0.01$ overall) as demonstrated in Table 2. No difference was seen in the number of patients receiving postoperative adjuvant chemotherapy between "front-positive" and "front-negative" groups ($P = 0.65$). Of the "front-positive" tumors, 37% were diagnosed before and 63% after 2005; the corresponding proportions for "front-negative" tumors being 22% and 78%, respectively ($P = 0.035$). No other significant correlations were seen between the staining pattern and clinicopathological variables, including PFS.

Table 2 Intratumoral staining pattern of membranous CD44 variant 6 expression related to treatment group and some clinicopathological variables *n* (%)

Variable (*n* = 177)	"Front-positive" (*n* = 105)	"Front-negative" (*n* = 72)	*P*[1]
Treatment group			
Short-course RT	46 (44)	32 (44)	
Long-course RT	24 (23)	14 (19)	0.87
Control	35 (33)	26 (36)	
Postoperative T			
T1-2	43 (41)	21 (29)	
T3	55 (52)	42 (58)	0.19
T4	7 (7)	9 (13)	
Postoperative N			
N0	68 (65)	40 (56)	
N1-2	36 (34)	31 (43)	0.37
Nx	1 (1)	1 (1)	
[2]Crm			
⩽ 2 mm	14 (18)	22 (38)	0.01
> 2 mm	64 (82)	36 (62)	
Tumor growth pattern			
Expanding	63 (60)	23 (32)	< 0.001
Infiltrating	42 (40)	49 (68)	
Disease-specific outcome			
Alive without recurrence	68 (65)	39 (54)	
Alive with recurrence	3 (3)	12 (17)	0.01
Died of disease	21 (20)	15 (21)	
Died of other causes	13 (12)	6 (8)	

[1]Pearson χ^2; *P* value for difference between "front-positive" and "front-negative" groups; [2]Circumferential margin, data available in 136 out of 177 cases. RT: Radiotherapy.

Table 3 Results of multivariate analysis

Variable	DFS			DSS		
	Adjusted HR	95% CI	*P*	Adjusted HR	95% CI	*P*
Sex						
Female (ref)	1.0			1.0		
Male	1.9	1.0-3.5	0.04	1.5	0.7-3.0	0.3
Age						
< 70 yr (ref)	1.0			1.0		
⩾ 70 yr	1.4	0.8-2.4	0.3	4.7	2.1-10.5	< 0.001
Postoperative T						
T1-2 (ref)	1.0			1.0		
T3	3.2	1.4-7.2	0.005	2.0	0.8-5.1	0.1
T4	5.9	2.0-17.0	0.001	4.8	1.4-17.0	0.02
Postoperative N						
N0 (ref)	1.0			1.0		
N1-2, Nx	2.0	1.1-3.7	0.02	0.8	0.4-1.7	0.6
[1]Postoperative grade						
G1 (ref)				1.0		
G2				1.3	0.4-3.9	0.7
G3				5.2	1.6-17.4	0.007
Growth pattern						
Expanding (ref)	1.0			1.0		
Infiltrating	1.0	0.5-1.8	0.9	0.8	0.4-1.7	0.6
[1]CD44v6 staining pattern						
"Front-positive" (ref)	1.0					
"Front-negative"	1.4	0.8-2.6	0.2			
Recurrence						
No (ref)				1.0		
Yes				74.3	17.0-326.7	< 0.001

[1]Not significant in univariate analysis and thus not included in multivariate model. Ref: Reference category; HR: Hazard ratio; DFS: Disease-free survival; DSS: Disease-specific survival; CD44v6: CD44 variant 6.

Recurrence and survival analysis

In univariate survival (Kaplan-Meier) analysis, no differences in DFS or DSS were seen according to the extent or intensity of CD44v6 expression, neither membranous nor cytoplasmic (data not shown). DFS ($P = 0.022$), but not DSS ($P = 0.68$), in patients with "front-negative" tumors was significantly shorter than in patients with "front-positive" tumors (Figure 2A and B). The same trend/difference was also seen in the short-course RT and control groups ($P = 0.058$ and $P = 0.024$ for difference in DFS, respectively) when analyzing treatment subgroups separately. With regard to tumor growth pattern, infiltrating tumors had shorter DFS ($P = 0.015$), and a tendency towards a shorter DSS ($P = 0.14$), as compared to expanding tumors.

The results of multivariate analysis are summarized in Table 3. The independent adverse prognostic factors for DFS were male sex, high postoperative T and the presence of positive lymph nodes, and those for DSS were patient age, postoperative T4, poor differentiation grade and disease recurrence.

DISCUSSION

Loss and gain of adhesive functions play an essential role in the progression of epithelial neoplasms[25]. In the present study, the expression of CD44v6, a cell surface mediator of cell-to-ECM adhesion[4], was systematically studied in a cohort of 214 primary rectal carcinomas treated with or without preoperative RT. The actual extent of CD44v6 expression was not associated with disease progression or outcome, but the analysis of its intratumoral staining pattern showed that patients with "front-negative" staining pattern had a significantly shorter DFS.

In our series, both membranous and cytoplasmic staining of CD44v6 was present in most cases, with a strong mutual correlation as reported earlier[8]. We found that preoperative RT affected cytoplasmic but not membranous CD44v6 expression. Although membranous CD44v6 is known to bind extracellular hyaluronate and growth factors[5], the biological significance of cytoplasmic CD44v6 is rather unknown[8]. In some studies it has been considered to have no functional roles[8], whereas in others, it has been related to neoplastic transformation[12,26] suggesting a role in loss of differentiation[26]. As preoperative RT has been shown to cause remarkable histological alterations[27], the reason for a decrease in cytoplasmic CD44v6 expression after RT could reflect these histological changes. However, further studies are required to elucidate this suggestion.

The prognostic value of CD44v6 expression, regarding the extent of expression, has been studied with inconclusive results in CRC[8,11,14]. In accordance with Morrin *et al*[14], we found no differences in disease progression indicators and patient outcome according to the

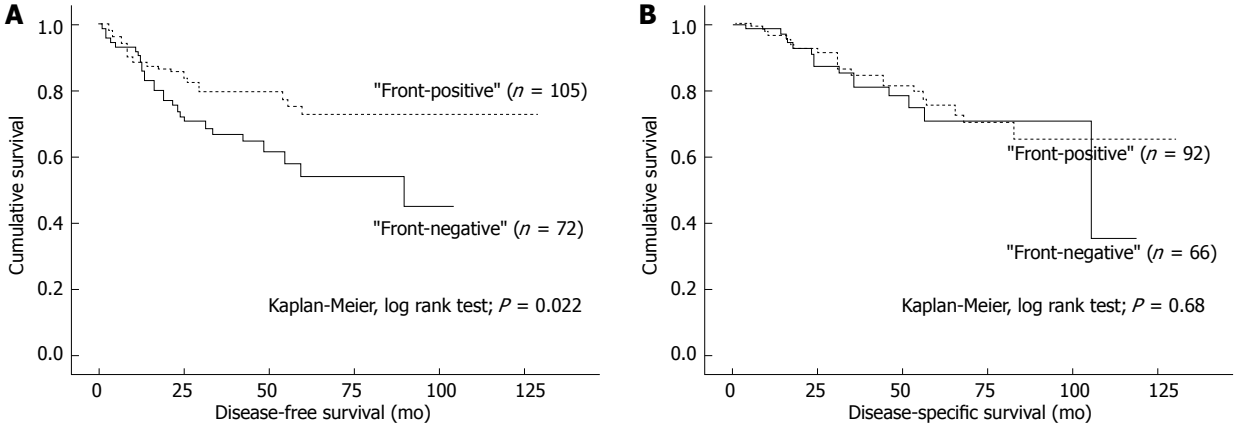

Figure 2 CD44 variant 6 related to disease-free and disease-specific survival in univariate analysis. Disease-free survival (A) and disease-specific survival (B) according to "front-positive" and "front-negative" membranous staining pattern of CD44 variant 6.

extent of CD44v6 expression. This finding is in contrast with some recent studies on rectal tumors, in which high membranous CD44v6 expression was shown to be associated with local recurrence[19] as well as shorter DFS[17-19] and DSS[17,18]. In contrast to these studies, we also included preoperatively treated tumors in our study to assess the possible relation of CD44v6 expression to TRG. This dissimilar study design may explain some of the discrepancy even though we found no differences in membranous CD44v6 expression parameters between the treatment or TRG-groups. In addition, the discrepancy may originate from differences in antibodies, cut-off points as well as disease subtypes in different studies as earlier discussed by Naor *et al*[28].

In addition to the extent of CD44v6 expression, we also assessed the intratumoral staining pattern of membranous expression. Given that tumor-host interaction at the invasive CRC front is thought to represent a dynamic interface between pro- and antitumor factors[20], it is not surprising that abnormalities in cell adhesion functions are seen in this compartment[29,30]. Although loss of membranous CD44v6 expression towards the CRC invasive front has been reported[8,12], our study is, to our knowledge, the first to assess the relation of membranous CD44v6 staining pattern to rectal cancer outcome. Furthermore, some of the previous studies are based on tissue microarray analyses, questioning the reliability of invasive front detection. Interestingly, "front-negative" tumors were related to more narrow postoperative circumferential margin and infiltrative tumor growth pattern as compared to "front-positive tumors" with apparent CD44v6 expression in the invasive front. Previously, Ishida[31] and Zlobec *et al*[8] with their co-workers have shown weak/negative CD44v6 expression to be related to infiltrating growth pattern, as assessed by its overall extent irrespective of its localisation. Our results may indicate that more important than the actual quantity of CD44v6 expression is, indeed, its localisation within the tumor. It is possible that "front-negative" tumors are more difficult for surgeon to resect with wide margins as they grow in a more diffuse manner, explaining the more narrow margins seen in these lesions.

Our observations lend support to the view of Zlobec *et al*[8], and Coppola *et al*[12], suggesting that the loss of membranous CD44v6 at the advancing edge of the tumor results in defective binding of the tumor cells to ECM, increasing their mobility and metastatic potential[8,12]. Further supporting this view, treatment with hyaluronic acid has recently been shown to delay the growth of residual colon carcinoma cells after chemotherapy[32]. Indeed, CD44v6 is known to have a higher affinity for hyaluronate, as compared to standard CD44[33], and, CD44v6-expressing tumors cells could be more effectively entrapped within the hyaluronic acid at the primary site[12]. In addition to binding with extracellular hyaluronate, membranous CD44v6 expression in the invasive front may also be important due to its intercellular adhesion properties, because loss of CD44v6 expression has been correlated to the loss of E-cadherin expression[8].

Importance of the localization of CD44v6 expression was further supported by the fact that "front-negative" tumors more often developed recurrent disease with shorter DFS as compared to patients with "front-positive" tumors. This was also observed in the subgroup analysis for the control group, and with a similar trend in the short-course RT group. No such appearance was seen in the long-course RT group, possibly due to the smaller number of cases available for this assessment. In some of the long-course RT cases, few tumor glands were left after treatment, and for this reason, no evident invasive front could be detected. However, despite this evident difference in DFS between the patients with "front-positive" and "front-negative" tumors, these two groups had practically identical DSS, which, due to the differences in the year of diagnoses between these two groups, could reflect the improvements in the treatment of recurrent rectal cancer.

The staining pattern of CD44v6 lost its significance in multivariate analysis. As in a number of other studies[34], we also found infiltrating tumor growth pattern to suggest an adverse DFS and a tendency towards a shorter DSS. The close correlation of CD44v6 staining

pattern with this prognostic factor may at least partly explain the lack of CD44v6 as an independent prognostic factor in our study. In addition, the other functions of this protein, such as binding of growth factors[35], may subvert the advantage of "front-positive" tumors.

Taken together, we found that tumors with "front-negative" CD44v6 membranous staining pattern were strongly related to invasive behavior. Our results substantiate the hypothesis that the lack, rather than overexpression, of membranous CD44v6 in the invasive tumor front contributes to rectal cancer progression. Although associated with a shorter DFS in univariate analysis, "front-negative" phenotype did not prove to be an independent prognostic factor in multivariate analysis, possibly confounded by other prognostic factors, including tumor growth pattern. We propose that analyzing the intratumoral staining pattern of membranous CD44v6 could offer a simple tool to predict the patients at increased risk for disease recurrence and thus in need of more aggressive postoperative treatment approaches and close monitoring.

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COMMENTS

Background

Colorectal cancer is a common malignancy. Radiotherapy alone or in combination with chemotherapy has been used to lower the risk of local recurrence. Still, a significant proportion of patients comes down with disease recurrence that is challenging to treat and causes harmful symptoms. New prognostic markers are constantly being studied to identify better the patients who need more aggressive treatment approaches in addition to surgery. CD44 variant 6 (CD44v6), a cell membrane receptor binding extracellular components, has been demonstrated to associate with rectal cancer progression and prognosis, but with discrepant results.

Research frontiers

In many studies, CD44v6 has been suggested to play a role in tumor progression and metastasis. The important area in the work was to study whether there is a difference in rectal cancer prognosis according to the intratumoral staining pattern of CD44v6. In contrast to previous studies, authors also included patients treated with radiotherapy before surgery.

Innovations and breakthroughs

The previous studies on the percentage and intensity of CD44v6 expression in rectal cancer have not been conclusive with regard to prognosis, because the overexpression of this protein has been associated with both favorable and adverse survival. Although alteration of CD44v6 expression in tumor invasive front has been demonstrated, its association with rectal cancer prognosis has not been studied in detail. Interestingly, the authors found that the patients with tumors showing CD44v6 expression in the invasive front presented with a longer disease-free survival time than did the patients whose tumors expressed protein more centrally. Moreover, the latter "front-negative" type was associated with infiltrating tumor growth and narrow tumor-free margin, both known to be adverse prognostic factors. Instead, the authors did not find the percentage and intensity of CD44v6 staining to be related to disease prognosis. The authors concluded that for rectal cancer progression and prognosis, more important than the actual amount of CD44v6 might be its distribution within the tumor.

Applications

The study suggests that patients with "front-negative" rectal cancer may need more aggressive treatment and monitoring after surgery.

Terminology

Prognostic factor is a situation or a condition, or a characteristic of a patient, that can be used to estimate the chance to recover from a disease or the risk for the disease recurring. Invasive front is the interface of tumor and host tissue; the deepest rim of cancerous tissue grown in adjacent non-cancerous tissues.

Peer review

In this work, the authors investigated the prognostic value of CD44v6 in patients treated with/without preoperative radiotherapy. The results are interesting and suggest that the lack of membranous CD44v6 in the rectal cancer invasive front could be used as a method to identify patients at increased risk for recurrent disease. The article is generally well written.

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BRIEF ARTICLE

Characteristics of intestinal pseudo-obstruction in patients with mitochondrial diseases

Yusuke Sekino, Masahiko Inamori, Eiji Yamada, Hidenori Ohkubo, Eiji Sakai, Takuma Higurashi, Hiroshi Iida, Kunihiro Hosono, Hiroki Endo, Takashi Nonaka, Hirokazu Takahashi, Tomoko Koide, Yasunobu Abe, Eiji Gotoh, Shigeru Koyano, Yoshiyuki Kuroiwa, Shin Maeda, Atsushi Nakajima

Yusuke Sekino, Masahiko Inamori, Eiji Yamada, Hidenori Ohkubo, Eiji Sakai, Takuma Higurashi, Hiroshi Iida, Kunihiro Hosono, Hiroki Endo, Takashi Nonaka, Hirokazu Takahashi, Tomoko Koide, Yasunobu Abe, Shin Maeda, Atsushi Nakajima, Gastroenterology Division, Yokohama City University School of Medicine, Yokohama 236-0004, Japan
Eiji Gotoh, Department of Medical Education, Yokohama City University School of Medicine, Yokohama 236-0004, Japan
Shigeru Koyano, Yoshiyuki Kuroiwa, Department of Neurology, Yokohama City University School of Medicine, Yokohama 236-0004, Japan
Author contributions: Sekino Y, Inamori M, Iida H, Nonaka T and Nakajima A performed the majority of clinical and treatment data collection; Takahashi H, Koide T, Abe Y, Gotoh E, Kuroiwa Y and Maeda S were involved in the editing of the manuscript; Yamada E, Ohkubo H, Sakai E, Higurashi T, Hosono K and Endo H analyzed the clinical data and participated in the design and coordination of the study; Sekino Y, Inamori M, Iida H, Nonaka T, Koyano S and Nakajima A designed the study and wrote the manuscript.
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Correspondence to: Dr. Masahiko Inamori, Gastroenterology Division, Yokohama City University School of Medicine, 3-9 Fukuura, Kanazawa-ku, Yokohama 236-0004, Japan. inamorim@med.yokohama-cu.ac.jp
Telephone: +81-45-7872640 Fax: +81-45-7843546
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Abstract

AIM: To reveal the frequency, characteristics and prognosis of chronic intestinal pseudo-obstruction (CIP) in mitochondrial disease patients.

METHODS: Between January 2000 and December 2010, 31 patients (13 males and 18 females) were diagnosed with mitochondrial diseases at our hospital. We conducted a retrospective review of the patients' sex, subclass of mitochondrial disease, age at onset of mitochondrial disease, frequency of CIP and the age at its onset, and the duration of survival. The age at onset or at the first diagnosis of the disorder that led to the clinical suspicion of mitochondrial disease was also examined.

RESULTS: Twenty patients were sub-classified with mitochondrial encephalopathy with lactic acidosis and stroke-like episodes (MELAS), 8 with chronic progressive external ophthalmoplegia (CPEO), and 3 with myoclonus epilepsy associated with ragged-red fibers (MERRF). Nine patients were diagnosed with CIP, 8 of the 20 (40.0%) patients with MELAS, 0 of the 8 (0.0%) patients with CPEO, and 1 of the 3 (33.3%) patients with MERRF. The median age (range) at the diagnosis and the median age at onset of mitochondrial disease were 40 (17-69) and 25 (12-63) years in patients with CIP, and 49 (17-81) and 40 (11-71) years in patients without CIP. During the survey period, 5 patients (4 patients with MELAS and 1 with CPEO) died. The cause of death was cardiomyopathy in 2 patients with MELAS, cerebral infarction in 1 patient with MELAS, epilepsy and aspiration pneumonia in 1 patient with MELAS, and multiple metastases from gastric cancer and aspiration pneumonia in 1 patient with CPEO.

CONCLUSION: Patients with CIP tend to have disorders that are suspected to be related to mitochondrial diseases at younger ages than are patients without CIP.

Key words: Chronic intestinal pseudo-obstruction; Criteria; Mitochondrial disease; Mitochondrial encephalopathy; Lactic acidosis; Stroke-like episodes; Chronic progressive external ophthalmoplegia

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INTRODUCTION

Intestinal pseudo-obstruction was first reported by Dudley *et al*[1] in 1958, and refers to an uncommon disabling motility syndrome characterized by severe symptoms and signs of intestinal obstruction (abdominal pain, abdominal distention, nausea, and vomiting) and radiographic evidence of a dilated bowel in the absence of any mechanical obstruction. Pseudo-obstruction is primarily considered a small-intestine motility disorder, but it may occur in any portion of the gastrointestinal tract[2-6]. Furthermore, pseudo-obstruction may occur as an acute disease (Ogilvie syndrome[7]) or as a chronic remitting or persistent disorder, and chronic intestinal pseudo-obstruction (CIP) can be caused by and complicate many disorders[2]. Primary CIP can be sub-classified into visceral myopathy, visceral neuropathy and idiopathic CIP based on its histopathological manifestations. According to its etiology, secondary CIP can be categorized as disease-induced (such as connective tissue disorders, muscular dystrophies, infiltrative diseases, mitochondrial diseases, generalized nerve disease, endocrine disease, metabolic disease and others)[8-12] or drug-induced (antidepressant and anti-anxiety drugs, phenothiazines and others)[13-16].

Although an algorithm for the diagnosis of CIP was proposed by Rudolph *et al*[2] in 1997, diagnostic criteria for CIP have not yet been established in Japan or worldwide. Recently, Iida *et al*[17] proposed the diagnostic criteria for CIP shown in Table 1 and reported a diagnostic sensitivity of 85.9%.

Mitochondrial diseases are a heterogeneous group of disorders associated with mutations or deletions of nuclear or mitochondrial DNA[18-22]. Genetic mutations or deletions result in multisystem involvement associated with defects in the oxidative phosphorylation system and impaired production of ATP. The degree of organ dysfunction is contingent on the energy requirement of the organ and the proportion of mutated mitochondrial DNA in the organ[18]. Encephalomyopathy and cardiomyopathy are frequently encountered manifestations of mitochondrial diseases, and recently, gastrointestinal dysmotility has received attention[23-26]. Chinnery *et al*[27] reported that over 15% of patients with mitochondrial diseases complain of dysphagia or constipation, and a small percentage of the cases with constipation develop intestinal pseudo-obstruction. Although Amiot *et al*[28] reported that 19% of 80 patients with CIP had mitochondrial defects, the relationship between CIP and mitochondrial disease has not yet been conclusively established.

The aim of this study was to determine the frequency, characteristics, and prognosis of CIP in patients with mitochondrial diseases.

MATERIALS AND METHODS

Between January 2000 and December 2010, 33 patients were diagnosed with mitochondrial diseases at the Yokohama City University School of Medicine. Their clinical and treatment data were collected from their medical records and 2 patients were excluded due to insufficient clinical data.

Diagnosis of mitochondrial disease

The plasma and cerebrospinal fluid levels of lactate and pyruvate were measured at rest and then re-evaluated under exercise stress to ensure that the levels were within the normal ranges. Muscle biopsies and genetic analyses were performed in all patients. Examinations for other component disorders of mitochondrial dysfunction, such as glucose intolerance, electrocardiography, echocardiography, and brain magnetic resonance imaging were performed as needed.

Ethical approval

This study was conducted in accordance with the declaration of Helsinki, and with the approval of the Ethics Committee of Yokohama City University School of Medicine. We obtained written informed consents from each of the patients.

Statistical analysis

We conducted a retrospective review of each patient's sex, subclass of mitochondrial disease, age at the onset of mitochondrial disease, age at the establishment of the diagnosis of mitochondrial disease, frequency of CIP and the age at onset, and the duration of survival. The age at onset or at the first diagnosis of the disorder that led to the clinical suspicion of mitochondrial disease was also examined. As controls, we collected the data of 57 patients who were diagnosed with progressive muscular dystrophy at the Yokohama City University School of Medicine between January 2007 and December 2011. Five of the patients were excluded due to insufficient clinical data.

RESULTS

Thirty-one patients with mitochondrial diseases underwent detailed assessment (Table 2). The subjects comprised 13 males and 18 females and were sub-classified as having mitochondrial encephalopathy with lactic acidosis and stroke-like episodes (MELAS; $n = 20$), chronic progressive external ophthalmoplegia (CPEO; $n = 8$), or myoclonus epilepsy associated with ragged-red fibers

Table 1 Criteria for the diagnosis of chronic intestinal pseudo-obstruction

Must include
One or more symptoms of ileus[1] onset at least 6 mo prior to diagnosis
One or both of following for the last 12 wk: (1) Abdominal pain; (2) Abdominal bloating
Dilatation and/or air-fluid levels of the intestine on abdominal X-ray, echo and/or computed tomography imaging
No evidence of structural disease (including findings of upper endoscopy, lower endoscopy, computed tomography, barium enema, and small-bowel follow-through) that could explain dilatation and/or air-fluid levels of the intestine
Supportive criteria
Congenital and/or onset under 15 years old must be excluded. Only adult onset is included
Surgical history within the 6 mo prior to diagnosis must be excluded to rule out Ogilvie syndrome, except surgery for CIP
To define CIP at two levels: Primary CIP or secondary CIP. Primary CIP consists of three types: the muscular type, neurogenic type and idiopathic type; Secondary CIP consists of two types: the systemic sclerosis (SSc) type and unclassified type
Family accumulation may exist
Neuropathy such as problems with urination may exist
Some psychosocial disorder may be present

[1]Symptoms of ileus include: Abdominal pain, nausea, vomiting, abdominal bloating, abdominal fullness, lack of defecation and/or passing gas. CIP: Chronic intestinal pseudo-obstruction.

Table 2 Age at the oncet of mitochondrial disease and chronic intestinal pseudo-obstruction

Sex	Subclass	Age at the onset of mitochondrial disease, yr	Age at the diagnosis of mitochondrial disease, yr	Age at the diagnosis of CIP, yr
F	MELAS	Difficulty in walking at 40	40	40
M	MELAS	Glucose intolerance at 13	57	46
M	MERRF	Difficulty in walking at 41	50	50
F	CPEO	Palpebral ptosis at 71	81	-
F	MELAS	Impaired hearing at 45	54	-
M	MELAS	Impaired hearing at 24	29	-
M	CPEO	Palpebral ptosis at 40	69	-
F	CPEO	Impaired eye movement at 30	52	-
F	MELAS	Impaired hearing at 25	40	43
M	CPEO	Palpebral ptosis at 49	49	-
F	MERRF	Muscular weakness at 46	54	-
M	MELAS	Impaired hearing at 47	52	-
M	MELAS	Epilepsy at 11	18	-
M	CPEO	Glucose intolerance at 40	67	-
M	MELAS	Impaired hearing at 24	31	-
F	MELAS	Impaired hearing at 12	17	25
F	MELAS	Impaired hearing at 54	60	-
F	MELAS	Epilepsy at 40	44	-
M	MELAS	Glucose intolerance and impaired hearing at 18	18	-
F	MELAS	-	23	-
F	MELAS	Chronic diarrhea at 26	40	-[1]
F	MELAS	Epilepsy at 18	18	26
F	MELAS	Difficulty in walking and hearing at 63	69	69
F	MELAS	Glucose intolerance at 33	42	55
M	CPEO	Palpebral ptosis at 16	44	-
M	MELAS	Epilepsy at 29	29	-
F	CPEO	Palpebral ptosis at 13	17	-
F	CPEO	Palpebral ptosis at 59	59	-
M	MELAS	Epilepsy at 21	21	34
F	MELAS	Glucose intolerance at 46	48	-
F	MERRF	Difficulty in walking at 20	50	-

[1]This patient died of uncontrolled epilepsy and aspiration pneumonia 5 mo after the onset of intestinal pseudo-obstruction and did not fulfill the diagnostic criteria. M: Male; F: Female; CIP: Chronic intestinal pseudo-obstruction; MERRF: Myoclonus epilepsy associated with ragged-red fibers; MELAS: Mitochondrial encephalopathy with lactic acidosis and stroke-like episodes; CPEO: Chronic progressive external ophthalmoplegia.

(MERRF; n = 3). Of the 31 patients, 9 (28.1%) were diagnosed with CIP based on Nakajima's criteria, including 8 of the 20 (40.0%) patients with MELAS, 0 of the 8 (0.0%) patients with CPEO, and 1 of the 3 (33.3%) patients with MERRF. One patient died of uncontrolled epilepsy and aspiration pneumonia 5 mo after the onset of intestinal pseudo-obstruction and did not fulfill the diagnostic criteria.

The confirmable outcomes were as follows: in December 2010, 14 patients were receiving outpatient treatment, 12 patients had been transferred to chronic care facilities, and 5 patients (4 patients with MELAS and 1 with CPEO) had died. The cause of death was cardiomyopathy in 2 patients with MELAS, cerebral infarction in 1 patient with MELAS, epilepsy and aspiration pneumonia in 1 patient with MELAS, as previously described, and multiple metastases from gastric cancer and aspiration pneumonia in 1 patient with CPEO.

In radiographic examinations for the 9 patients with CIP, small intestinal distention was observed in 6 patients, and large intestinal distention was observed in 1 patients. Both small and large intestinal distention was observed in 2 patients.

The median age (range) at the establishment of the diagnosis of mitochondrial disease was 40 (17-69) years in patients with CIP and 49 (17-81) years in patients without CIP. The median age (range) at the onset or at the first diagnosis of the disorder that led to the suspicion of mitochondrial disease was 25 (12-63) years in patients with CIP and 40 (11-71) years in patients without CIP.

The symptoms of CIP were treated with laxative agents, antidiarrheal drugs, antiflatulence agents, mosapride, dimethicone, pantothenic acid, daikenchuto (Chinese herbal medicine), neostigmine or distigmine, and none of the patients required surgery (Table 3).

As controls, we collected the data of 57 patients with progressive muscular dystrophy (Table 4). The patients consisted of 2 patients with Duchenne muscular dystrophy, 7 patients with Becker muscular dystrophy, 4

Table 3 Characteristics of chronic intestinal pseudo-obstruction in patients with mitochondrial diseases

Sex	Subclass	Type of abdominal discomfort	Area of dilated intestine	Treatment
F	MELAS	Nausea, vomiting, diarrhea, constipation	Small intestine	PEG, neostigmine
M	MELAS	Diarrhea	Small intestine	Antidiarrheal drug
M	MERRF	Pain, diarrhea, distension	Small and large intestines	Mosapride, dimethicone, antiflatulent, daikenchuto, pantothenic acid
F	MELAS	Diarrhea, constipation, distension	Small and large intestines	Mosapride, daikenchuto, magnesium oxide
F	MELAS	Nausea, vomiting, diarrhea, constipation, pain	Small intestine	PEG, antiflatulent, magnesium oxide, antidiarrheal drug
F	MELAS	Nausea, vomiting, constipation, pain	Small intestine	Prostarmon, magnesium oxide, dimethicone, daikenchuto, mosapride, neostigmine, sodium picosulfate hydrate
F	MELAS	Nausea, vomiting, distension	Small intestine	Magnesium oxide, daikenchuto
F	MELAS	Nausea, vomiting	Large intestine	Daikenchuto, sennoside
M	MELAS	Nausea, vomiting, diarrhea, distension	Small intestine	Daikenchuto, mosapride, magnesium oxide, lansoprazole, neostigmine, dimechicone

M: Male; F: Female; CIP: Chronic intestinal pseudo-obstruction; MERRF: Myoclonus epilepsy associated with ragged-red fibers; MELAS: Mitochondrial encephalopathy with lactic acidosis and stroke-like episodes; PEG: Polyethylene glycol.

Table 4 Characteristics of chronic intestinal pseudo-obstruction in patients with progressive muscular dystrophy

Subclass	No. of patients	No. of females	Age at the onset[1]	Age at the diagnosis[1]
Duchenne muscular dystrophy	2	0	1	2 (1-3)
Becker muscular dystrophy	7	0	10 (1-30)	19 (6-43)
Limb-girdle muscular dystrophy	4	1	22.5 (5-40)	49 (10-53)
Facio-scapulo-humeral muscular dystrophy	1	1	NS	NS
Myotonic dystrophy	38	18	30 (5-62)	39 (12-67)
Total	52	20	25 (1-62)	34.5 (1-67)

[1]Data is presented as median age (range). NS: Not stated. None of the patients with progressive muscular dystrophy in this study fulfilled the diagnostic criteria of chronic intestinal pseudo-obstruction.

patients with limb-girdle muscular dystrophy, 1 patient with facio-scapulo-humeral muscular dystrophy and 43 patients with myotonic dystrophy (5 patients were excluded due to insufficient clinical data). The median age (range) at the establishment of the diagnosis of progressive muscular dystrophy was 34.5 (1-67) years and the median age (range) at the onset of progressive muscular dystrophy was 25 (1-62) years. Only 2 of the patients died, while the others were alive at the last hospital visit. Although progressive muscular dystrophy has been considered as one of the etiologies of CIP, none of the patients with progressive muscular dystrophy in this study fulfilled the diagnostic criteria of CIP.

DISCUSSION

Although mitochondrial diseases have been considered as a potential cause of secondary CIP, the frequency of CIP in patients with mitochondrial diseases has not yet been well established. Gastrointestinal dysmotility (including CIP) may be caused by the accumulation of intracellular long-chain fatty acids, activation of extramitochondrial fatty acid oxidation pathways and excessive generation of reactive oxygen species leading to visceral myopathy, possibly as a result of impaired mitochondrial beta-oxidation[29]. Betts *et al*[30] reported that profound COX deficiency was found in the smooth muscle layers of all regions of the gastrointestinal tract in patients with the m.3243A>G mutation despite scarce evidence of morphologic abnormalities within the gastrointestinal tissues of these patients. Parsons *et al*[31] also reported that 80% of patients with MELAS and more than 60% of m.3243A>G carriers have 1 or more autonomic symptoms; gastrointestinal symptoms were especially common in the MELAS group, occurring in 66% of these patients and in almost 40% of the mutation carriers. In mitochondrial diseases, mitochondrial neurogastrointestinal encephalopathy (MNGIE), which is an uncommon autosomal recessive syndrome caused by the reduced activity of thymidine phosphorylase due to a mutation of the nuclear DNA, has also been reported to be particulary associated with CIP along with other symptoms such as malnutrition, progressive external ophthalmoplegia, ptosis, peripheral neuropathy, and leukoencephalopathy[32-34]. Although we have never encountered patients with MNGIE, this study revealed that patients with mitochondrial diseases develop complicating CIP at a relatively high frequency (28.1%), especially patients with MELAS (40.0%). We also examined the clinical data of patients with progressive muscular dystrophy, which is also a known cause of CIP, as controls and found that CIP occurs less frequently in patients with progressive muscular dystrophy than in patients with mitochondrial diseases (0% *vs* 28.1%).

Patients with CIP tend to have disorders, such as glucose intolerance, epilepsy, hearing impairment and palpebral ptosis,that are suspected to be related to mitochondrial diseases at younger ages than are patients without CIP. None of the patients in our study presented with CIP as the initial symptom of mitochondrial disease. This trend indicates that some patients with mitochondrial dysfunction will develop gastrointestinal dysmotility with disease progression, eventually leading

to the development of CIP.

The frequency of CIP among patients with relatively mild mitochondrial dysfunction who do not need advanced treatment at a hospital may also be smaller than that noted in this study. Most patients with mitochondrial diseases die of cardiomyopathy and encephalopathy before the development of severe CIP, and this finding is consistent with a previous report indicating that the degree of organ dysfunction depended on the energy requirement of the organ. Although CIP was not a major cause of death in patients with mitochondrial diseases, it often resulted in impaired oral intake or tube feeding and eventually the critical loss of the activities of daily life. Early detection and treatment of gastrointestinal dysmotility, including CIP, and the maintenance of the nutritional status may be efficacious in the management of patients with mitochondrial diseases.

There is still no sufficiently effective treatment for CIP today. A number of medicines were used in this study, and none were especially effective when comparing these treated patients with CIP patients without mitochondrial diseases. Surgery has been reported to be effective in patients with normal small intestinal motility[35], although surgery was not performed in this study (8 of 9 patients with CIP in this study had small intestine dysmotility).

Selection bias is one of the major limitations of our study. Patients receiving outpatient treatment at a university hospital like ours may have more severe symptoms than general patients presenting to primary care practitioners may have. This limitation could have resulted in an over-estimation of the CIP prevalence in patients with mitochondrial diseases. In addition, the 31 patients with mitochondrial diseases in this study included 20 patients with MELAS (64.5%), 8 patients with CPEO (25.8%) and 3 patients with MERRF (9.7%), indicating a larger prevalence of MELAS patients in our study compared with that from an epidemiological study conducted in Japan (64.5% *vs* 25.5%)[36]. This selection bias may also have contributed to an over-estimation of the CIP prevalence. Moreover, patients with MELAS often use antiepileptic drugs regularly, which may cause drug-induced intestinal pseudo-obstruction. In our study, 5 patients with CIP regularly used antiepileptic drugs (phenytoin, carbamazepine, carbamazepine and sodium valproate, and for 2 patients, carbamazepine and clonazepam). Although it may be difficult to precisely distinguish between mitochondrial and drug-induced gastrointestinal symptoms, the regular use of these antiepileptic drugs alone rarely results in CIP, and mitochondrial dysfunction was considered the main cause of the severe intestinal dysmotility of these 5 patients.

In conclusion, patients with mitochondrial diseases (especially MELAS) sometimes develop CIP. In cases characterized by the adequate control of fetal mitochondrial dysfunctions such as cardiomyopathy and encephalopathy, CIP may present as a prominent problem. The possibility of the development of gastrointestinal dysmotility, including CIP, should be considered at all stages of the clinical course of all patients with mitochondrial diseases.

COMMENTS

Background

Chronic intestinal pseudo-obstruction (CIP) is characterized by severe symptoms and signs of intestinal obstruction without mechanical obstruction. Although mitochondrial diseases have been considered as a potential cause of secondary CIP, the frequency, characteristics, and prognosis of CIP in patients with mitochondrial diseases has not yet been well established.

Research frontiers

Mitochondrial diseases are a heterogeneous group of disorders associated with mutations in or deletions of nuclear or mitochondrial DNA. The degree of organ dysfunction depends on the energy requirements of the organ and on the proportion of mitochondrial DNA in the organ. In the treatment of mitochondrial patients with CIP, current research now focuses on how mitochondrial dysfunction develops into CIP and what the differences between the patients with CIP and without CIP are.

Innovations and breakthroughs

The relationship between CIP and mitochondrial diseases has not yet been firmly established. In the present study, authors conducted a retrospective review of each patient's sex, subclass of mitochondrial disease, age at the onset of mitochondrial disease, age at the establishment of the diagnosis of mitochondrial disease, frequency of CIP and the age at its onset, and the duration of survival. The age at the onset or at the first diagnosis of the disorder that led to the clinical suspicion of mitochondrial disease was also examined.

Applications

The study results suggest that patients who have certain disorders, such as glucose intolerance, epilepsy, hearing impairment and palpebral ptosis, that are suspected to be related to mitochondrial diseases at younger ages tend to develop gastrointestinal dysmotility with disease progression, eventually leading to the development of CIP.

Terminology

CIP is an uncommon disabling motility syndrome characterized by severe symptoms and signs of intestinal obstruction (abdominal pain, abdominal distention, nausea and vomiting) and radiographic evidence of dilated bowels in the absence of any mechanical obstruction; Mitochondrial diseases are a heterogeneous group of disorders associated with mutations in or deletions of nuclear or mitochondrial DNA.

Peer review

This is a good descriptive study in which the authors retrospectively reviewed the frequency, characteristics and prognosis of chronic intestinal pseudo-obstruction in mitochondrial patients. The study revealed that patients with CIP tend to have disorders that are suspected to be related to mitochondrial diseases at younger ages than patients without CIP.

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BRIEF ARTICLE

Effect of composite yogurt enriched with acacia fiber and *Bifidobacterium lactis*

Yang Won Min, Sang Un Park, Yeon Sil Jang, Young-Ho Kim, Poong-Lyul Rhee, Seo Hyun Ko, Nami Joo, Sun Im Kim, Cheol-Hyun Kim, Dong Kyung Chang

Yang Won Min, Sang Un Park, Yeon Sil Jang, Young-Ho Kim, Poong-Lyul Rhee, Dong Kyung Chang, Division of Gastroenterology, Department of Medicine, Samsung Medical Center, Sungkyunkwan University School of Medicine, Seoul 135-710, South Korea
Seo Hyun Ko, Nami Joo, Sun Im Kim, Department of Food and Nutrition, Sookmyung Women's University, Seoul 140-742, South Korea
Cheol-Hyun Kim, Department of Animal Resource and Science, Dankook University, Cheonan 330-714, South Korea
Author contributions: Min YW analyzed the data and wrote the draft manuscript; Park SU and Jang YS analyzed the data; Kim YH and Rhee PL provided the data and undertook critical revision of the manuscript; Ko SH, Joo N, Kim SI and Kim CH undertook critical revision of the manuscript and were involved in study design; and Chang DK designed the study.
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Correspondence to: Dong Kyung Chang, Professor, MD, PhD, Division of Gastroenterology, Department of Medicine, Samsung Medical Center, Sungkyunkwan University School of Medicine, 50 Irwon-dong, Gangnam-gu, Seoul 135-710, South Korea. dkchang@skku.edu
Telephone: +82-2-34103409 Fax: +82-2-34106983
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Abstract

AIM: To investigate whether composite yogurt with acacia dietary fiber and *Bifidobacterium lactis* (*B. lactis*) has additive effects in irritable bowel syndrome (IBS).

METHODS: A total of 130 patients were randomly allocated to consume, twice daily for 8 wk, either the composite yogurt or the control product. The composite yogurt contained acacia dietary fiber and high-dose *B. lactis* together with two classic yogurt starter cultures. Patients were evaluated using the visual analog scale *via* a structured questionnaire administered at baseline and after treatment.

RESULTS: Improvements in bowel habit satisfaction and overall IBS symptoms from baseline were significantly higher in the test group than in the control group (27.16 *vs* 15.51, $P = 0.010$, 64.2 ± 17.0 *vs* 50.4 ± 20.5, $P < 0.001$; respectively). In constipation-predominant IBS, improvement in overall IBS symptoms was significantly higher in the test group than in the control group (72.4 ± 18.4 *vs* 50.0 ± 21.8, $P < 0.001$). In patients with diarrhea-predominant IBS, improvement in bowel habit satisfaction from baseline was significantly higher in the test group than in the control group (32.90 *vs* 7.81, $P = 0.006$).

CONCLUSION: Our data suggest that composite yogurt enriched with acacia fiber and *B. lactis* has greater therapeutic effects in patients with IBS than standard yogurt.

Key words: Acacia dietary fiber; *Bifidobacterium lactis*; Irritable bowel syndrome; Probiotics; Yogurt

Peer reviewers: Dr. Ian David Wallace, Shakespeare Specialist Group,181 Shakes-perare Rd., Milford, 1309 Auckland, New Zealand; Gabrio Bassotti, Professor, Clinical and Experimental Medicine, Gastroenterology and Hepatology Section, Ospedale Santa Maria della Misericordia, Piazza Menghini, 06156 Perugia, Italy

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INTRODUCTION

Irritable bowel syndrome (IBS) is a functional bowel disorder characterized by symptoms of abdominal pain or discomfort associated with disturbed defecation, and is one of the most common gastrointestinal problems[1-3]. The pathogenesis of IBS is incompletely understood but over the past few years, there has been an emergence of new etiological hypotheses. These include gastrointestinal infection, low-grade infiltration and activation of mast cells in the intestinal mucosa with consequent release of bioactive substances, modification of small bowel and colonic microflora, changes related to the brain-gut axis, and altered serotonin metabolism[4-8]. These new views of the pathogenesis of IBS have changed the approach to IBS treatment[9]. Among several treatment options, the use of probiotics seems to be promising[10].

Probiotics are live microorganisms with a vast array of therapeutic potential for gastrointestinal disease[11,12]. They have been studied and used in many gastrointestinal disorders, with growing evidence for use in pouchitis, *Clostridium difficile* colitis, antibiotic-associated diarrhea, inflammatory bowel disease, and IBS. The emerging multifactorial pathophysiological paradigm of IBS may create adjunctive probiotic therapeutic opportunities[10,13,14]. The most widely studied organisms are *Bifidobacterium lactis* (*B. lactis*) and *Lactobacillus spp.*[15]. *B. lactis* survives complete transit through the digestive tract and is recovered live in stools in large quantities relative to the quantity initially ingested[16,17]. Daily consumption of fermented milk containing *B. lactis* was reported to improve gastrointestinal transit and digestive comfort, alleviate bloating, and increase stool frequency[18,19].

Acacia gum is extensively used as a food additive. It is a complex polysaccharide, that is primarily indigestible, not degraded in the intestine, but fermented in the colon[20]. Acacia fiber is made from acacia gum. Recently, its prebiotic properties, meaning it selectively stimulates the intestinal flora, were described and a synergy for bifidogenicity was observed with the combination of other prebiotics (fructo-oligosaccharide) and acacia gum. In addition, because acacia fiber is slowly fermented, it may attenuate the side effects of fermentation. Intestinal gas production resulting from fermentation can induce abdominal symptoms[21]. Dietary fiber is also commonly used in the treatment of patients with IBS[22]. Although dietary fiber does not appear to be useful as a sole treatment of IBS, it may have a limited role in empiric therapy, especially if constipation is the most significant symptom[23,24].

The aim of this study was to investigate whether a composite yogurt enriched with acacia dietary fiber and *B. lactis* had additive therapeutic effects in patients with IBS when compared with standard yogurt.

MATERIALS AND METHODS

Study population

A total of 130 patients were recruited at Samsung Medical Center, Seoul, South Korea. They were male and female patients between 18 and 70 years of age who met the Rome Ⅲ criteria[25] for the diagnosis of IBS.

Exclusion criteria were as follow: (1) endocrine disorders, neurological disorders, cardiovascular disorders, inherited neuromuscular disorders, malignant tumors, renal failure (serum creatinine ≥ 3.0 mg/dL), and liver cirrhosis (child class B and C); (2) current use of medications that potentially influence bowel habits such as medications for constipation, antidiarrheal drugs, and medications that can cause constipation, including anticholinergic drugs (e.g., anticonvulsants, antihistamines, antipsychotic and neuroleptic agents, anti-Parkinson agents, and antidepressants), narcotic pain medications (e.g., codeine), resins (e.g., cholestyramine), and metal ions and inorganic compounds (e.g., aluminum-, calcium-, and iron-containing antacids); (3) age > 55 years without a history of a sigmoidoscopy or colonoscopy performed in the previous 5 years; (4) abnormal results on a sigmoidoscopy or colonoscopy and abdominal radiological tests performed in the previous 2 years; and (5) any previous abdominal surgery.

Study protocol

This was a randomized, double-blind, controlled trial. Simple randomization was performed using a random number table. Potentially eligible patients answered a structured questionnaire at baseline. At that time, they were provided with a standardized explanation of questions and symptom definitions. In addition, patients were evaluated *via* a full review of their clinical history including complete blood count, serum chemistry, and thyroid function test. Clinically significant abnormalities in any of the latter tests resulted in exclusion from the study. Thereafter, eligible patients were randomly allocated to consume two bottles daily (one at breakfast, one at dinner) for 8 wk of either the composite yogurt (test product) or the control product. At the end of the study, patients were again administered a questionnaire. The use of any medication that potentially influences bowel habits was prohibited for 7 d prior to consumption of the products.

The study protocol was conducted in accordance with the Declaration of Helsinki and approved by the Institutional Review Board at Samsung Medical Center, Seoul, South Korea (No. 2010-07-223). All subjects provided written informed consent before inclusion in the study.

Study products

The test product was a yogurt containing high-dose *Bifidobacterium animalis subsp. lactis Bb-12* (*B. animalis subsp. lactis Bb-12*) (≥ 10^{11} cfu/bottle), *Bifidobacterium* enhancer, and acacia dietary fiber, together with the two classic yogurt starter cultures, *Streptococcus thermophilus* (≥ 3×10^9 cfu/bottle) and *Lactobacillus acidophilus* (*L. acidophilus*) (≥ 10^9 cfu/bottle).

The control product was a traditional yogurt contain-

ing *B. animalis subsp. lactis Bb-12* ($\geq 10^{10}$ cfu/bottle), no extra-functional ingredients (*Bifidobacterium* enhancer and acacia dietary fiber), and two yogurt starter cultures, *Streptococcus thermophilus* ($\geq 3 \times 10^{9}$ cfu/bottle) and *L. acidophilus* ($\geq 1 \times 10^{9}$ cfu/bottle). Both the test and control products were without added flavor and had similar appearance, color, texture, and taste. Each bottle contained either 150 mL of test or control product and was provided by the Namyang Dairy Products Co. Ltd. (Seoul, South Korea).

Assessments

Each patient was evaluated using a structured questionnaire at baseline and after 8 wk treatment. At baseline, the questionnaire assessed age, sex, height, body weight, IBS subtype[26], abdominal symptoms (abdominal pain/discomfort, abdominal distension/bloating, and flatulence), and bowel habits (frequency and duration of defecation, urgency, straining, feeling of incomplete defecation, stool consistency, bowel habit satisfaction, and discomfort related to daily life). Abdominal pain/discomfort, abdominal distension/bloating, bowel habit satisfaction, and discomfort related to daily life were evaluated using the visual analog scale (VAS, 0 = no symptoms, 25 = mild, 50 = moderate, 75 = severe, and 100 = very severe). Abdominal pain/discomfort, flatulence, and defecation were assessed by frequency. The stool consistency was determined using the Bristol stool scale[27].

At the end of the treatment, abdominal symptoms and bowel habits were assessed by the self-administration of the questionnaire that had the same questions as the one at baseline. In addition, the improvement of overall IBS symptoms was evaluated using the VAS (0 = aggravated, 25 = no change, 50 = slightly improved, 75 = much improved, 100 = very much improved).

Statistical analysis

It was determined that 50 patients per group were required for a power of 85% and two-sided significance at 5% in detecting a between-group effect of 0.2 in the improvement of overall IBS symptoms. To ensure the inclusion of at least 50 patients per group, 65 per group were ultimately recruited in order to account for a potential withdrawal rate of 25%.

Baseline demographic data were compared between groups using Student's *t* test, Pearson's χ^2 test, or Fisher's exact test, as appropriate. Changes in the symptom scores after treatment for each group were assessed using a paired *t* test, McNemar's test, and a generalized estimating equation. A two-sided *P* value < 0.05 was considered statistically significant. Statistical analysis was performed using PASW Statistics 18 for Windows (SPSS, Inc, Chicago, IL, United States).

RESULTS

Baseline characteristics and response to treatment

A total of 130 patients were enrolled and randomized either to the test group ($n = 65$) or the control group ($n = 65$). Thirteen patients discontinued the study and were lost to follow-up, and 117 (58 in the test group and 59 in the control group) completed the study. Using the Rome Ⅲ criteria, 35.0% of patients were classified as constipation-predominant IBS (IBS-C), 29.9% as diarrhea-predominant IBS (IBS-D), 8.5% as mixed IBS (IBS-M), and 26.5% as unsubtyped IBS. Table 1 shows baseline characteristics and symptom scores of patients in the test and control groups. At baseline, the distributions of age, sex, body mass index, and IBS subtype were similar between the groups, and the baseline scores for abdominal symptoms and bowel habits did not differ.

Table 2 summarizes the changes in the study parameters after 8 wk treatment in both the test and control groups and differences between the groups. Bowel habit satisfaction improved more in the test group than in the control group (change from baseline of 27.16 *vs* 15.51, $P = 0.010$), and the improvement in overall IBS symptoms was significantly higher in the test group than in the control group (64.2 ± 17.0 *vs* 50.4 ± 20.5, $P < 0.001$). The scores for abdominal pain/discomfort, abdominal distension/bloating, and discomfort related to daily life also improved more in the test group than in the control group, but the improvements did not significantly differ between the groups. The improvements in straining and feeling of incomplete evacuation did not differ between the groups. Defecation duration and frequency, flatulence, and urgency did not improve after treatment. The change in stool consistency was different in the two groups. Stool consistency did not change in the test group but became softer in the control group. There were no significant adverse events reported throughout the study.

Analysis by IBS subtype

A subgroup analysis was performed to determine the effects of the test product on each IBS subtype. In the IBS-C group (Table 3), the improvement in overall IBS symptoms was significantly higher in the test group than in the control group (72.4 ± 18.4 *vs* 50.0 ± 21.8, $P < 0.001$), and the difference between the two groups was greater than that between the test and control groups including all of the study patients (64.2 ± 17.0 *vs* 50.4 ± 20.5, $P < 0.001$). However, bowel habit satisfaction did not differ between the two groups. Defecation frequency and feeling of incomplete evacuation, which did not improve in the study patients overall, improved in the test and control groups (change from baseline of 1.79, $P = 0.002$ and 1.96, $P = 0.032$; change from baseline -42.1%, $P = 0.021$ and -31.8%, $P = 0.016$, respectively) although the improvements did not significantly differ between the groups. Stool consistency became softer in both groups without a significant difference between the test and control groups (change from baseline of 0.789 *vs* 1.09, $P = 0.386$). In the IBS-D group (Table 4), bowel habit satisfaction improved more in the test group than in the control group (change from baseline of 32.90 *vs*

Table 1 Baseline characteristics and symptom scores (mean ± SD)

	Test group (n = 58)	Control group (n = 59)	P value
Age (yr)	37.43 ± 10.27	34.24 ± 8.67	0.720
Male:female n (%)	17 (29.3):41 (70.7)	18 (30.5):41 (69.5)	0.887
BMI (kg/m^2)	21.96 ± 3.01	21.28 ± 2.50	0.184
Subtype of IBS n (%)			0.919
Constipation	19 (32.8)	22 (37.3)	
Diarrhea	19 (32.8)	16 (27.1)	
Mixed	5 (8.6)	5 (8.5)	
Unsubtyped	15 (25.9)	16 (27.1)	
Abdominal symptoms			
Abdominal pain or discomfort (VAS)	34.05 ± 18.55	33.05 ± 19.94	0.779
Frequency of abdominal pain or discomfort/d	1.73 ± 2.03	1.21 ± 1.17	0.089
Abdominal distension or bloating (VAS)	44.40 ± 21.99	39.83 ± 20.82	0.281
Flatulence/d	5.19 ± 4.01	4.98 ± 2.88	0.749
Bowel habit			
Defecation frequency/wk	6.38 ± 5.98	5.69 ± 3.87	0.458
Defecation duration (min)	8.68 ± 6.35	8.85 ± 5.59	0.881
Urgency n (%)	23 (39.7)	22 (37.3)	0.792
Straining n (%)	39 (67.2)	37 (62.7)	0.608
Feeling of incomplete defecation n (%)	39 (67.2)	44 (74.6)	0.382
Stool consistency (BSS)	3.95 ± 1.64	3.54 ± 1.59	0.159
Bowel habit satisfaction (VAS)	32.33 ± 17.53	31.95 ± 18.22	0.909
Discomfort related to daily life (VAS)	36.21 ± 22.54	30.93 ± 17.58	0.160

BMI: Body mass index; IBS: Irritable bowel syndrome; VAS: Visual analog scale; BSS: Bristol stool scale.

Table 2 Study parameters at week 8 and changes from baseline in all patients (mean ± SD)

	Test group (n = 58)				Control group (n = 59)				P value[1]
	Baseline	wk 8	Δ wk 8	P value	Baseline	wk 8	Δ wk 8	P value	
Abdominal symptoms									
Abdominal pain or discomfort (VAS)	34.05 ± 18.55	12.93 ± 14.99	-21.12	0	33.05 ± 19.94	16.5 ± 17.7	-16.53	< 0.001	0.26
Frequency of abdominal pain or discomfort/d	1.73 ± 2.03	0.84 ± 0.83	-0.89	0.004	1.21 ± 1.17	0.7 ± 0.8	-0.48	0.001	0.214
Abdominal distension or bloating (VAS)	44.40 ± 21.99	25.86 ± 18.1	-18.53	0	39.83 ± 20.82	28.8 ± 21.2	-11.02	< 0.001	0.096
Flatulence/d	5.19 ± 4.01	5.69 ± 5.08	0.5	0.391	4.98 ± 2.88	5.5 ± 4.0	0.51	0.266	0.991
Bowel habits									
Defecation frequency/wk	6.38 ± 5.98	7.23 ± 4.28	0.85	0.289	5.69 ± 3.87	6.7 ± 4.2	1.04	0.052	0.843
Defecation duration (min)	8.68 ± 6.35	7.47 ± 4.95	-1.22	0.07	8.85 ± 5.59	6.7 ± 3.9	-2.12	< 0.001	0.301
Urgency	23 (39.7%)	16 (27.6%)	-7	0.118	22 (37.3%)	22 (37.3%)	0	1	0.146
Straining	39 (67.2%)	22 (37.9%)	-17	0	37 (62.7%)	20 (33.9%)	-17	0.002	0.959
Feeling of incomplete evacuation	39 (67.2%)	19 (32.8%)	-20	0	44 (74.6%)	23 (39.0%)	-21	< 0.001	0.815
Stool consistency (BSS)	3.95 ± 1.64	3.72 ± 1.02	-0.22	0.274	3.53 ± 1.59	3.88 ± 1.15	0.36	0.047	0.118
Bowel habit satisfaction (VAS)	32.33 ± 17.53	59.48 ± 19.21	27.16	0	31.95 ± 18.22	47.5 ± 20.1	15.51	< 0.001	0.01
Discomfort related to daily life (VAS)	36.21 ± 22.54	21.98 ± 19.91	-14.22	0	30.93 ± 17.58	22.9 ± 17.5	-8.05	0.007	0.199
Improvement in overall IBS symptoms (VAS)		64.2 ± 17.0				50.4 ± 20.5			< 0.001

[1]Comparison between both groups. IBS: Irritable bowel syndrome; VAS: Visual analog scale; BSS: Bristol stool scale.

7.81, P = 0.006), and the difference between the two groups was greater than the difference between patients in the overall analysis (change from baseline of 27.16 *vs* 15.51, P = 0.010). However, the improvement in overall IBS symptoms did not differ between the two groups. Abdominal pain/discomfort scores improved more in the test group than in the control group, and the improvement was nearly significant (change from baseline of -23.68 *vs* -9.38, P = 0.050). Stool consistency became harder in both groups without a significant difference between the test and control groups (change from baseline of -1.26 *vs* -0.63, P = 0.738). In the IBS-M group, the improvements in abdominal symptoms, bowel habits, and overall IBS symptoms did not significantly differ between the test and control groups.

DISCUSSION

Several clinical studies have demonstrated the efficacy of probiotics for the treatment of patients with IBS. Some studies have also been performed in populations of certain IBS subtypes. A study by Kim *et al*[28] evaluated the effect of VSL#3, a combination of probiotics that contains live bacteria including *Bifidobacterium*, *Lactobacillus*, and *Streptococcus salivarius ssp. thermophilus,* on gastrointestinal transit and symptoms in IBS-D. With VSL#3 treatment, the decrease in bloating was of borderline significance, but there was no effect on gastrointestinal transit

Table 3 Study parameters at week 8 and changes from baseline in constipation-predominant irritable bowel syndrome patients (mean ± SD)

	Test group (n = 19)				Control group (n = 22)				P value[1]
	Baseline	wk 8	Δ wk 8	P value	Baseline	wk 8	Δ wk 8	P value	
Abdominal symptoms									
Abdominal pain or discomfort (VAS)	28.95 ± 20.90	9.21 ± 12.39	-19.74	0.001	37.50 ± 25.30	15.91 ± 18.17	-21.59	0.001	0.8
Frequency of abdominal pain of discomfort/d	1.50 ± 1.17	0.89 ± 0.88	-0.61	0.032	1.27 ± 1.56	0.68 ± 0.78	-0.6	0.029	0.979
Abdominal distension or bloating (VAS)	44.74 ± 21.38	25.00 ± 16.67	-19.74	0.007	38.64 ± 22.79	26.14 ± 16.33	-12.5	0.031	0.393
Flatulence/wk	6.05 ± 5.03	6.13 ± 4.35	0.08	0.952	5.52 ± 3.34	6.02 ± 4.95	0.5	0.577	0.785
Bowel habit									
Defecation frequency/wk	3.82 ± 2.08	5.61 ± 3.54	1.79	0.002	3.43 ± 1.55	5.39 ± 3.97	1.96	0.032	0.872
Defecation duration (min)	12.26 ± 7.33	9.66 ± 5.28	-2.61	0.106	10.84 ± 6.49	6.59 ± 4.07	-4.25	< 0.001	0.358
Urgency	4 (21.1%)	4 (21.1%)	0	1	3 (13.6%)	6 (27.3%)	3	0.375	0.336
Straining	18 (94.7%)	11 (57.9%)	-7	0.016	18 (81.8%)	12 (54.5%)	-6	0.146	0.321
Feeling of incomplete evacuation	15 (78.9%)	7 (36.8%)	-8	0.021	16 (72.7%)	9 (40.9%)	-7	0.016	0.776
Stool consistency (BSS)	2.26 ± 0.45	3.05 ± 0.85	0.789	0	2.18 ± 0.66	3.27 ± 1.08	1.09	0.001	0.386
Bowel habit satisfaction	30.26 ± 19.68	56.58 ± 20.14	26.32	0	27.27 ± 18.76	44.32 ± 21.73	17.05	0.004	0.21
Discomfort related to daily life (VAS)	34.21 ± 22.38	18.42 ± 16.33	-15.79	0.014	36.36 ± 21.45	27.73 ± 15.25	-13.64	0.015	0.782
Improvement in overall IBS symptoms (VAS)		72.4 ± 18.4				50.0 ± 21.8			< 0.001

[1]Comparison between both groups. IBS: Irritable bowel syndrome; VAS: Visual analog scale; BSS: Bristol stool scale.

Table 4 Study parameters at week 8 and changes from baseline in diarrhea-predominant irritable bowel syndrome patients (mean ± SD)

	Test group (n = 19)				Control group (n = 16)				P value[1]
	Baseline	wk 8	Δ wk 8	P value	Baseline	wk 8	Δ wk 8	P value	
Abdominal symptoms									
Abdominal pain or discomfort (VAS)	32.89 ± 14.56	9.21 ± 14.93	-23.68	0	29.69 ± 10.08	20.3 ± 18.8	-9.38	0.083	0.05
Frequency of abdominal pain or discomfort/d	2.45 ± 3.18	0.63 ± 0.83	-1.82	0.036	1.25 ± 0.71	0.91 ± 0.94	-0.34	0.245	0.117
Abdominal distension or bloating (VAS)	43.42 ± 23.34	25.00 ± 20.41	-18.42	0.012	35.94 ± 15.73	29.69 ± 20.85	-6.25	0.164	0.146
Flatulence/wk	4.63 ± 2.36	4.08 ± 2.67	-0.55	0.503	4.66 ± 2.15	5.50 ± 3.38	0.84	0.255	0.212
Bowel habit									
Defecation frequency/wk	10.55 ± 8.18	8.79 ± 4.46	-1.76	0.381	9.09 ± 4.19	9.09 ± 4.12	0	1	0.451
Defecation duration (min)	6.66 ± 5.57	6.58 ± 4.98	-0.08	0.938	8.00 ± 4.75	7.03 ± 3.81	-0.97	0.3	0.52
Urgency	10 (52.6%)	8 (42.1%)	-2	0.625	12 (75.0%)	11 (68.8%)	-1	1	0.867
Straining	8 (42.1%)	3 (15.8%)	-5	0.063	7 (43.8%)	2 (12.5%)	-5	0.063	0.707
Feeling of incomplete evacuation	13 (68.4%)	6 (31.6%)	-7	0.039	12 (75.0%)	5 (31.3%)	-7	0.016	0.826
Stool consistency (BSS)	5.42 ± 1.17	4.16 ± 0.38	-1.26	0.001	5.50 ± 1.10	4.88 ± 1.09	-0.63	0.036	0.738
Bowel habit satisfaction	31.58 ± 18.34	64.47 ± 19.21	32.9	0	40.63 ± 15.48	48.44 ± 17.00	7.81	0.173	0.006
Discomfort related to daily life (VAS)	36.84 ± 24.11	23.68 ± 25.65	-13.16	0.163	29.69 ± 13.60	28.13 ± 17.97	-1.56	0.751	0.292
Improvement in overall IBS symptoms (VAS)		61.8 ± 17.4				51.6 ± 14.3			0.07

[1]Comparison between both groups. IBS: Irritable bowel syndrome; VAS: Visual analog scale; BSS: Bristol stool scale.

or other individual IBS symptoms. A study by Guyonnet *et al*[19] assessed the effects of fermented milk containing *B. animalis* DN-173 010 in IBS-C and reported improvements in the health-related quality-of-life discomfort score and bloating symptoms, as well as increased stool frequency. Another study demonstrated the effects of a fermented milk product containing *B. lactis* DN-173-010 on abdominal distension and gastrointestinal transit in IBS-C[29]. In the present study, the therapeutic effect of the composite yogurt differed according to IBS subtype; in IBS-C, overall IBS symptoms were improved, and in IBS-D, the improvement in bowel habit satisfaction was prominent. However, irrespective of IBS subtype, the new composite yogurt had additive therapeutic effects on bowel habit satisfaction and overall IBS symptoms among the entire IBS study sample when compared with standard yogurt.

Lactobacilli and *bifidobacteria*, alone or in combination, have been used for the treatment of IBS in many clinical studies. A study by Sinn *et al*[14] reported that 4 wk treatment with *L. acidophilus*-SDC 2012, 2013 was associated with a reduced score for abdominal pain or discomfort compared to baseline. O'Mahony *et al*[30] performed a study in patients with IBS and grouped them into three different treatment arms. The patients received *Lactobacillus salivarius* UCC4331, *Bifidobacterium infantis* (*B. infantis*) 35624, or placebo, but only *B. infantis* alleviated IBS symptoms. This was associated with a normalization of the anti-inflammatory to proinflammatory cytokine ratio (interleukin-10/interleukin-12), suggesting an immune-modulating role for *B. infantis*. A larger study by Whorwell *et al*[31] evaluating different doses of *B. infantis* 35624 was performed in 362 women with IBS. The participants were randomized to receive either the placebo or encapsulated *B. infantis* at a dose of 10^6, 10^8 or 10^{10} cfu for 4 wk. *B. infantis* at a dose of 10^8 cfu was significantly superior to placebo and all other doses in improving IBS symptoms. However, the 10^{10} dose was associated

with significant formulation problems. From only these two studies, it is not possible to determine which strain is most effective and the optimal dose. Nevertheless, to date, *Bifidobacterium* seems to be one of the most important probiotics, and a *B. infantis* dose of at least 10^8 cfu may be appropriate for the treatment of IBS. Given this fact, it can be deduced that at a *Bifidobacterium* dose > 10^8 cfu, the therapeutic effects would increase in proportion to the dose up to a certain point. In this study, we used *B. lactis*, which has been shown to have therapeutic effects in IBS in several studies[18,19,29,32]. Our results also showed that the new composite yogurt (containing > 10 times the *B. lactis* of the control product and *Bifidobacterium* enhancer) was associated with a significant improvement in bowel habit satisfaction and overall IBS symptoms, although acacia fiber was also added to the test product and it was difficult to attribute the results to one or the other component. To determine the optimal dosage, another trial evaluating the effects of yogurt containing *B. lactis* at different dosages is necessary.

Dietary fiber accelerates whole gut transit time and increases daily stool weight and the proportion of unformed stool, and its efficacy in alleviating constipation has been confirmed in patients with IBS[33]. Therefore, dietary fiber is frequently recommended for IBS[22]. A study by Choi *et al*[24] evaluated the additive effects of probiotic fermented milk containing dietary fiber in IBS-C patients, compared to plain probiotic fermented milk, and dietary fiber had additive benefits for the symptoms of constipation, especially in IBS-C. However, in a study by Francis *et al*[34], fiber was found to exacerbate all symptoms of IBS. Dietary fiber is classified into soluble and insoluble fiber, which have different effects on global IBS-related symptoms[35]. Soluble fiber delays gastric emptying and nutrient absorption from the small bowel; it is used to delay gastric emptying and improve glycemic control in diabetes, as well as to alleviate constipation. Insoluble fiber has little effect on gastric emptying and small bowel transit; it markedly accelerates colonic transit and is frequently used as a laxative[36]. For our study, we used acacia fiber, which is soluble, and that is thought to be the reason the composite yogurt containing acacia fiber was associated with improvement in IBS symptoms among IBS-C patients.

The present study had some limitations. First, the follow-up period was relatively short. It would have been useful to know how participants were faring after using the composite yogurt for 6 and 12 mo. Next, this study was limited by the lack of a placebo group because the control product was not a placebo. Finally, we did not investigate participants' dietary factors, which may be more prevalent in IBS-D. Nevertheless, this was a well-designed trial with an appropriate number of patients. Furthermore, additive effects of high-dose *B. lactis* and acacia dietary fiber were clearly noted with respect to bowel habit satisfaction and overall IBS symptoms. Among IBS subtypes, overall IBS symptoms were more improved in IBS-C; in IBS-D, bowel habit satisfaction was more improved. In conclusion, a new composite yogurt had greater therapeutic effects in patients with IBS than standard yogurt had. Further studies are needed to determine the most effective probiotic strain, dosage, and duration of therapy.

COMMENTS

Background

Irritable bowel syndrome (IBS) is one of the most common gastrointestinal problems. The new views of the pathogenesis of IBS have changed the approach to IBS treatment. Among several treatment options, the use of probiotics seems to be promising. Dietary fiber is also commonly used in the treatment of patients with IBS.

Research frontiers

The authors investigated whether a composite yogurt enriched with acacia dietary fiber and *Bifidobacterium lactis* (*B. lactis*) had additive therapeutic effects in patients with IBS when compared with standard yogurt.

Innovations and breakthroughs

This study was a well-designed prospective clinical trial with an appropriate number of patients. Additive effects of acacia dietary fiber and high-dose *B. lactis* were clearly noted with respect to bowel habit satisfaction and overall IBS symptoms.

Applications

The study results suggest that composite yogurt enriched with acacia fiber and *B. lactis* has greater therapeutic effects in patients with IBS than standard yogurt has.

Terminology

Probiotics are live microorganisms with a vast array of therapeutic potential for gastrointestinal disease.

Peer review

This study is very informative for clinicians because IBS is one of the most common gastrointestinal problems and clinicians frequently encounter problems with treating IBS patients. In addition, the results have scientific relevance for understanding the pathogenesis of the disease.

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BRIEF ARTICLE

Reduction of gastrointestinal motility by unilateral thyroparathyroidectomy plus subdiaphragmatic vagotomy in rats

Jun Ho Lee, Oh Deog Kwon, Seon Ho Ahn, Keun Han Choi, Ji Hye Park, Seoul Lee, Bong Kyu Choi, Kyu Yong Jung

Jun Ho Lee, Keun Han Choi, Ji Hye Park, Seoul Lee, Bong Kyu Choi, Kyu Yong Jung, Department of Pharmacology, Wonkwang University School of Medicine, Jeonbuk 570-749, South Korea
Oh Deog Kwon, Department of Clinical Pathology, College of Veterinary Medicine, Kyungpook National University, Daegu 702-701, South Korea
Seon Ho Ahn, Department of Internal Medicine, Wonkwang University School of Medicine, Jeonbuk 570-749, South Korea
Author contributions: Lee JH conducted the surgery; Kwon OD performed the biochemistry and analyzed the data; Ahn SH designed the study; Choi KH analyzed the data; Park JH, Lee S and Choi BK compiled the data; Jung KY wrote the paper.
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Correspondence to: Kyu Yong Jung, DVM, PhD, Department of Pharmacology, Wonkwang University School of Medicine, 344-2 Shinyong-dong, Iksan, Jeonbuk 570-749, South Korea. wkuniv@wonkwang.ac.kr
Telephone: +82-63-8506796 Fax: +82-63-8506764
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Abstract

AIM: To investigate whether the combined methods of unilateral thyroparathyroidectomy (TPX) and subdiaphragmatic vagotomy (VAX) can be adapted for rats and used as a reliable method to produce a rat model of long-term reduction of gastrointestinal (GI) motor function.

METHODS: Male Sprague-Dawley rats were randomly divided into 3 groups, normal, sham-operated and unilateral TPX plus VAX. The TPX plus VAX rats received VAX 7 d after application of TPX, and dietary intake and fecal output were then measured daily for 1 wk. After completion of the experiments, gastric emptying and small bowel transit were measured *in vivo*, and the contractile responses of colonic strips to excitatory and inhibitory neurotransmitters were estimated using isometric force transducers *in vitro*.

RESULTS: In comparison with normal and sham-operated rats, rats which received unilateral TPX plus VAX showed a significant decrease in body weight and in fecal pellet number and weight throughout the entire week. Application of TPX plus VAX to rats markedly delayed gastric emptying and small bowel transit. In TPX plus VAX rats, the longitudinal muscles of the proximal colon showed a significant reduction in contractile responses to acetylcholine (5×10^{-6} mol/L), and a dramatic attenuation of contractile responses was also observed in both the longitudinal and circular muscles of the distal colon. However, the spontaneous contractility of the colonic strips from TPX plus VAX rats was not significantly affected by treatment with *N*-nitro-*L*-arginine-methyl ester (0.1 mol/L).

CONCLUSION: The results indicate that unilateral TPX plus VAX reduced the motor function of the GI tract in rats, and the reduced gut motility is likely mediated, at least in part, by inhibition of the excitatory neurotransmitter system.

Key words: Unilateral thyroparathyroidectomy; Subdiaphragmatic vagotomy; Gastric emptying; Small bowel transit; Rat

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INTRODUCTION

Vagal nerves and thyroid hormones regulate the physiological functions of the gastrointestinal (GI) tract. The subdiaphragmatic vagal nerve and its branches innervate the GI tract from the lower esophageal sphincter to the upper GI tract in humans[1,2], and the extensive distribution of vagal nerves to the gut plays an important role in the regulation of many digestive functions including food intake, gastric accommodation and GI motility[1,3]. Therefore, abnormalities in the subdiaphragmatic vagal nerves pathologically mediates the development of several GI diseases including dyspepsia and constipation[1,3,4]. In addition, hyper- and hypo-thyroidism also gives rise to a variety of GI symptoms including abnormal gastric secretion and GI motility[5,6]. The effects of hypothyroidism on the GI tract seem to be multi-factorial with alterations in neuroregulation, and a reduction in peristaltic activity being the most frequent GI complaint in patients with hypothyroidism[7].

It has been suggested that the vagal nerves predominantly act on the stomach and small intestine rather than the lower GI tract, and that subdiaphragmatic vagotomy (VAX) results in a transient slowing of the rate of gastric emptying and transit of the upper small intestine[8-10]. In our preliminary studies, rats which received subdiaphragmatic VAX transiently showed delayed gastric emptying and small bowel transit after surgery. Additionally, it has been reported that a complete depletion of thyroid hormones by bilateral thyroparathyroidectomy (TPX) in rats can induce a severe tetany which has a high mortality rate[11]. We also observed a similar phenomenon in preliminary studies, where bilateral TPX caused an extremely high fatality rate $\geqslant$ 90%) of rats within 48 h after surgical application. Taken together, we consider that bilateral TPX or subdiaphragmatic VAX alone is not a reliable and reproducible method to induce a long-term reduction in upper GI motility.

Therefore, we hypothesized that in comparison with bilateral TPX or subdiaphragmatic VAX, a combined method (TPX plus VAX) of unilateral TPX and subdiaphragmatic VAX might be a better strategy to provide a long-term reduction in spontaneous motility of the entire GI tract, but with reduced postoperative mortality. To examine this hypothesis, this study measured the effects of unilateral TPX plus VAX on the alteration of GI motility using rats, which are not only a popular laboratory animal for biomedical research, but also are a good model for studying the pharmacological properties of medicines, and for investigating the pathophysiological characteristics of human GI dysfunction[12,13].

MATERIALS AND METHODS

Animals and experimental groups

Male Sprague-Dawley rats (6-wk old) were obtained from Samtako BioKorea (Kyungki, South Korea) and acclimatized for 1 wk with free access to a solid normal rodent diet (Samyang Co., Kyungki, South Korea) and drinking water under a temperature of 23 ℃ ± 2 ℃, a relative humidity of 50%-60% and a 12-h/12-h dark-light cycle. After acclimatization, rats were randomly divided into 3 groups: normal, sham-operated and unilateral TPX plus VAX groups. Each group comprised 7-9 rats. The experimental procedures employed in this study are illustrated in Figure 1. All experiments were conducted in accordance with the institutional guidelines established by the Wonkwang University Commolittee for the Care and Use of Laboratory Animals.

Unilateral thyroparathyroidectomy

Unilateral TPX was carried out according to the previous method with a slight modification[14]. In brief, rats (7-wk old) were anesthetized using chloropent (5 mg/kg *ip*) for deep anesthesia, and thyroparathyroid glands on the right side were exposed by a mid-line incision of the neck under aseptic conditions and excised under stereomicroscopic observation. For sham-operated rats, the operation was performed using the same procedures except with no dissection of the thyroparathyroid glands. After surgery, animals were individually housed for 1 wk in cages with bedding of wood shavings.

Subdiaphragmatic vagotomy

At 7 d after unilateral TPX, subdiaphragmatic VAX was carried out according to a previous report with a slight modification[15]. Briefly, rats (8-wk old) were anesthetized using chloropent (5 mg/kg *ip*), and the abdomen around the stomach was opened under aseptic conditions. The ventral and dorsal branches of the subdiaphragmatic vagal nerves were cut (0.5 cm above the gastroesophageal junction). In sham-operated rats, the vagal trunks were similarly exposed, but without cutting of the vagal nerves. At the end of surgery, the completeness of VAX was verified by gastric responses to electrical stimulation, and inspection of the vagal nerve endings using a stereomicroscope as described in previous reports[8,15]. After dissecting the vagal nerves, animals were individually acclimatized for 1 d in wood-bedded cages and then housed in stainless-steel metabolic cages throughout the entire experimental period. Body weight, dietary intake, and fecal pellet number and weight were measured daily (9:00 am) for 7 d.

Measurement of gastric emptying

Gastric emptying was measured according to the previous method with a slight modification[16]. At 8 d after subdiaphragmatic VAX, rats were starved overnight but with drinking water available *ad libitum*. Each animal was sacrificed by cervical dislocation 10 min after oral ad-

ministration of a test meal (1 mL) containing 0.05% (w/v) phenol red in aqueous carboxymethyl cellulose (4.5%, w/v), and the stomach was immediately removed, followed by clamping of the gastro-esophageal and gastro-duodenal junctions. The stomach was rinsed with phosphate-buffered saline (PBS, pH 7.4) and dissected in 20 mL 0.1 mol/L NaOH. The stomach, containing phenol red solution, was shaken for 30 min and centrifuged at 3000 *g* for 10 min. To precipitate proteins, 0.5 mL of supernatant was added to 20% trichloroacetic acid (50 mL) and mixed vigorously. The samples were centrifuged at 10 000 *g* for 30 min, and 0.3 mL supernatant was mixed with 0.5 mol/L NaOH (0.4 mL) to develop the color. The absorbance of samples at 558 nm wavelength was measure with a spectrophotometer (U-2000; Hitachi, Tokyo, Japan). Gastric emptying was estimated from the following formulation: (amount of residual phenol red/phenol red present in stomach immediately after administration) × 100.

Measurement of small bowel transit

To examine the spontaneous motility of the small intestine, a small bowel transit test was measured an established method[17]. Briefly, rats at 8 d after application of subdiaphragmatic VAX were starved overnight but with drinking water available *ad libitum*. Each animal was orally administered 1 mL of a charcoal meal, 10% (w/v) activated charcoal in aqueous Arabic gum (5%, w/v), and sacrificed by cervical dislocation 20 min later. The gastro-duodenal and ileo-cecal junctions were tied, and the entire small intestine was then quickly removed to avoid stretching. After rinsing the intestine with PBS, small bowel transit was estimated by comparing the distance travelled by the charcoal meal from the pyloric sphincter to the total length of the small intestine from the pyloric sphincter to the ileo-cecal junction. The distance travelled by the charcoal meal was expressed as a percentage of the total length of the small intestine.

Preparation of colonic strips

At 8 d after subdiaphragmatic VAX, rats were starved overnight but with drinking water available *ad libitum* and sacrificed by CO_2 asphyxiation and cervical dislocation. The entire proximal and distal colons were removed, and the luminal contents were flushed out using Krebs-Ringer bicarbonate buffer (composition 118 mmol NaCl, 4.7 mmol KCl, 1.2 mmol KH_2PO_4, 1.2 mmol $MgSO_4$, 2.6 mmol $CaCl_2$, 25 mmol $NaHCO_3$ and 11.5 mmol *D*-glucose, pH 7.4). Strips (1 cm length) of proximal colon at a distance of 1 cm from the ileo-cecal junction and distal colon and at a distance of 1 cm from the anus were dissected. The intact colonic strips were longitudinally or circularly mounted in an organ bath (10 mL) containing Krebs-Ringer bicarbonate buffer bubbled with 5% CO_2/95% O_2 and maintained at 37 ℃. One end of each colonic strip was tied with suture silk and fixed to the bottom of the organ bath, and the other end was connected to an isometric force transducer (Grass Technologies, West Warwick, RI).

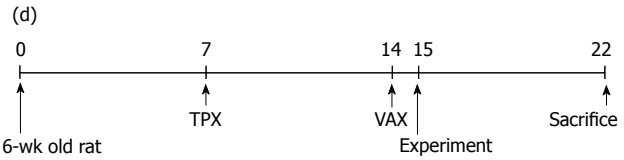

Figure 1 Schematic diagram of experiments to examine gastrointestinal motility after sequential application of unilateral thyroparathyroidectomy and subdiaphragmatic vagotomy. TPX: Thyroparatyroidectomy; VAX: Subdiaphragmatic vagotomy.

Measurement of colonic contractility

Colonic contractility was measured as described in our previous report[18]. Briefly, after the colonic strips were allowed to equilibrate for 30-60 min with washout every 10 min, 1 g of tension was slowly applied to the tissues before starting the experiments. The strips were treated with 0.5 mol of acetylcholine (ACh) or 0.1 mmol of *N*-nitro-*L*-arginine methyl ester (L-NAME) for 10 min, and the contractile responses of the colonic strips prepared from the unilateral TPX plus VAX rats to ACh and L-NAME were estimated and compared with the colonic contractility in strips of normal animals. Isometric contractile responses from the force transducer were measured by a biological recording system equipped with an amplifier (PowerLab 4/25, AD Instruments, Colorado Springs, CO, United States). The mean tensions and amplitudes of colonic contraction produced under resting and drug-treated conditions were measured over a period of 10 min.

Measurement of serum thyroxine contents

To estimate the change of serum thyroxine (T_4) level caused by unilateral TPX, total serum T_4 level at 15 d after application of unilateral TPX were measured using the T_4 enzyme-linked immunosorbent assay kit (GenWay Biotech; San Diego, CA, United States). Blood samples were collected from the abdominal vein at the time of sacrifice, and serum was obtained by centrifugation at 3000 *g* for 10 min. Serum samples were stored at -70 ℃ until T_4 analysis.

Statistical analysis

All results are presented as mean ± SE. One-way analysis of variance was performed to compare the statistical significance of multi-groups, followed by *post hoc* analysis by the Student-Newman-Keuls test for comparison of 2 groups. $P < 0.05$ was considered statistically significant.

RESULTS

General observation and serum thyroxine levels

No deaths occurred in the rats which received unilateral TPX plus VAX. In these rats, an obvious dilatation of the stomach and significant residual luminal content in the GI tract were found, but marked morphological changes

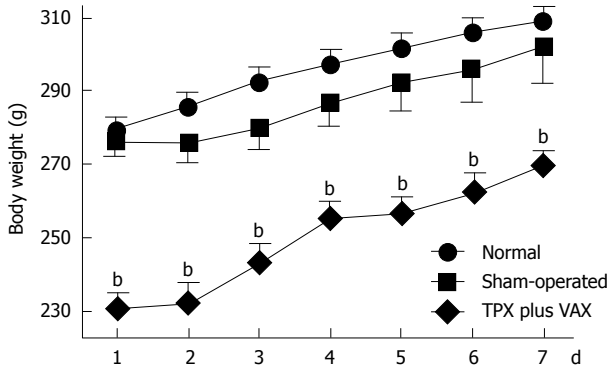

Figure 2 Effects of unilateral thyroparatyroidectomy plus subdiaphragmatic vagotomy on body weight gain in rats. Body weights were measured for 7 d from the day after subdiaphragmatic vagotomy (VAX). Each point represents the mean ± SE of 7-9 rats. $^{b}P < 0.01$ *vs* normal and sham-operated groups. TPX: Thyroparatyroidectomy.

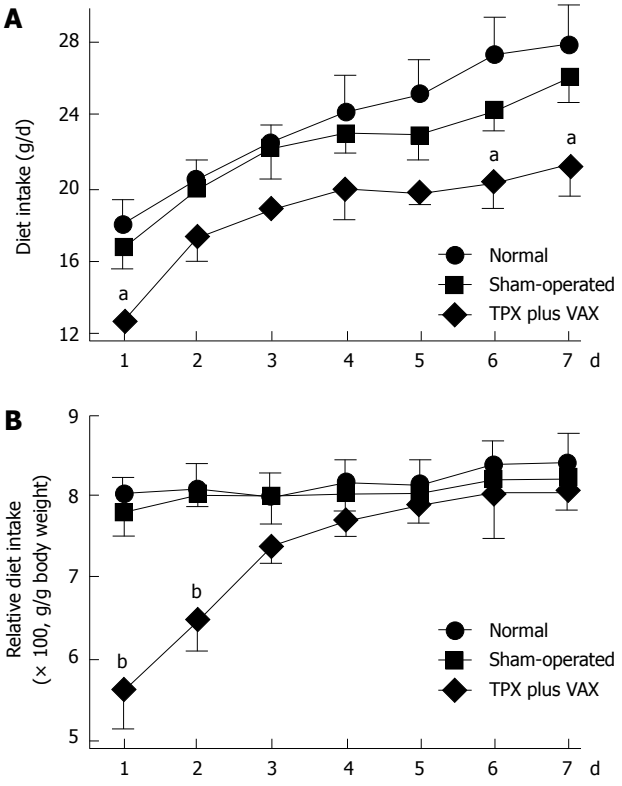

Figure 3 Changes in daily dietary intake in the thyroparatyroidectomy plus subdiaphragmatic vagotomy rats. Daily dietary intake (A: Absolute; B: Relative to body weight) was measured for 7 d from the day after subdiaphragmatic vagotomy (VAX). Each point represents the mean ± SE of 7-9 rats. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* normal and sham-operated groups. TPX: Thyroparatyroidectomy.

of the gut were not observed. There were no significant differences in visible features and behaviors among the normal (4.74 ± 0.85 g/dL), sham-operated (4.97 ± 1.14 g/dL) and unilateral TPX plus VAX rats (2.86 ± 0.98 g/dL). Serum T_4 levels in the unilateral TPX plus VAX rats were not significantly lower than those in the normal and sham-operated animals.

Body weight and dietary intake

As shown in Figure 2, body weights of unilateral TPX plus VAX rats were significantly lower than those of normal and sham-operated animals throughout the entire experimental period. However, there was no significant difference in body weights between normal and sham-operated rats. Daily dietary intake of the unilateral TPX plus VAX rats was not significantly lower than in normal and sham-operated animals throughout the entire experimental period (Figure 3A). The relative daily dietary intake in unilateral TPX plus VAX rats transiently decreased, but almost completely returned to normal levels after 4 d (Figure 3B).

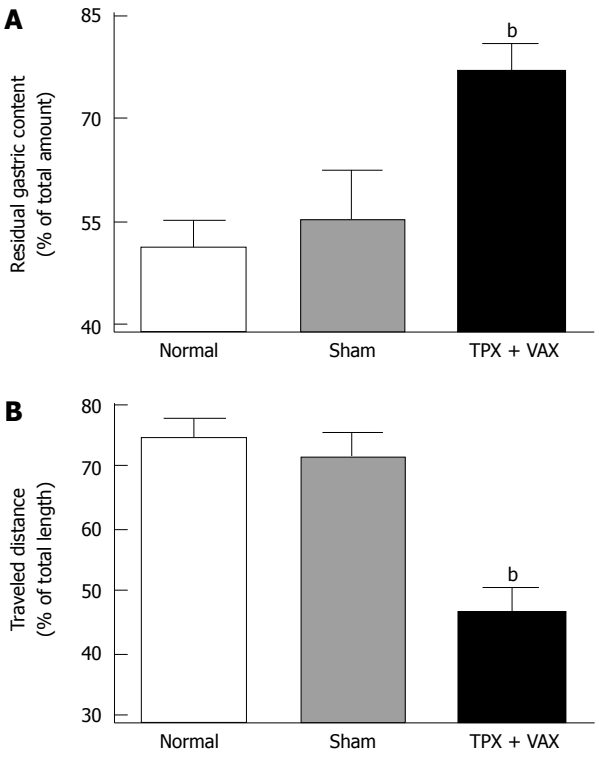

Figure 4 Effects of thyroparatyroidectomy plus subdiaphragmatic vagotomy on gastric emptying and small bowel transit in rats. Gastric emptying (A) and small bowel transit (B) was measured 8 d after subdiaphragmatic vagotomy (VAX). Each point represents the mean ± SE of 7-9 rats. $^{b}P < 0.01$ *vs* normal and sham-operated groups. TPX: Thyroparatyroidectomy.

Gastric emptying and small bowel transit

The motor functions of upper GI tracts were estimated by the gastric emptying test and small bowel transit *in vivo*, and the obtained results are shown in Figure 4. In comparison with normal and sham-operated rats, the residual gastric contents of unilateral TPX plus VAX rats were significantly elevated (Figure 4A), and the traveled distances of the luminal content in the small intestines of unilateral TPX plus VAX rats were markedly shortened (Figure 4B).

Defecatory function

Defecatory function was determined by measuring fecal pellet numbers and weights, and the obtained results are shown in Figure 5. In comparison with normal and sham-operated rats, daily fecal pellet numbers of unilateral TPX plus VAX rats were significantly reduced

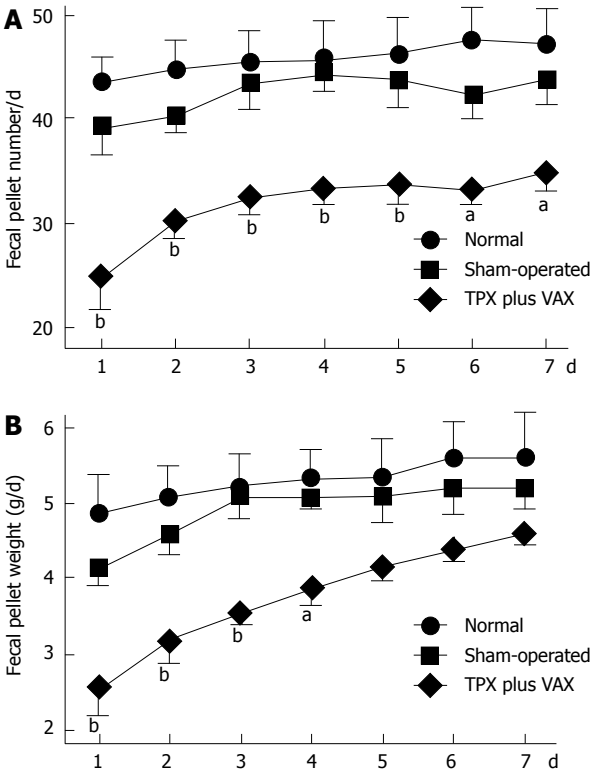

Figure 5 Effects of thyroparatyroidectomy plus subdiaphragmatic vagotomy on defecation of rats. Fecal pellet numbers (A) and weights (B) were measured for 7 d from the day after subdiaphragmatic vagotomy (VAX). Each point represents the mean ± SE of 7-9 rats. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* normal and sham-operated groups. TPX: Thyroparatyroidectomy.

throughout the entire experimental periods (Figure 5A), and their daily fecal weights were markedly reduced for the first 4 d of the experimental period (Figure 5B).

Colonic contractility

To examine changes in the spontaneous contractility of colonic smooth muscles, the contractile responses of intact colonic strips to ACh (0.5 mol) and L-NAME (0.5 mmol) were measured. The representative tracings obtained for proximal and distal colons are shown in the Figures 6A and 7A, respectively. In comparison with normal rats, the contractile responses to ACh (0.5 mol), both tension and amplitude, of the proximal colon longitudinal muscles prepared from the unilateral TPX plus VAX rats were significantly attenuated, whereas no significant changes were observed in the circular muscles (Figure 6B). L-NAME-induced contractility of the proximal colon did not significantly differ between the normal and unilateral TPX plus VAX rats (Figure 6C). Additionally, ACh-induced contractions of the distal colonic longitudinal and circular muscles prepared from the unilateral TPX plus VAX rats were significantly reduced compared with those of normal animals (Figure 7B), whereas the contractile responses of colonic strips to L-NAME did not significantly differ between normal and unilateral TPX plus VAX rats (Figure 7C).

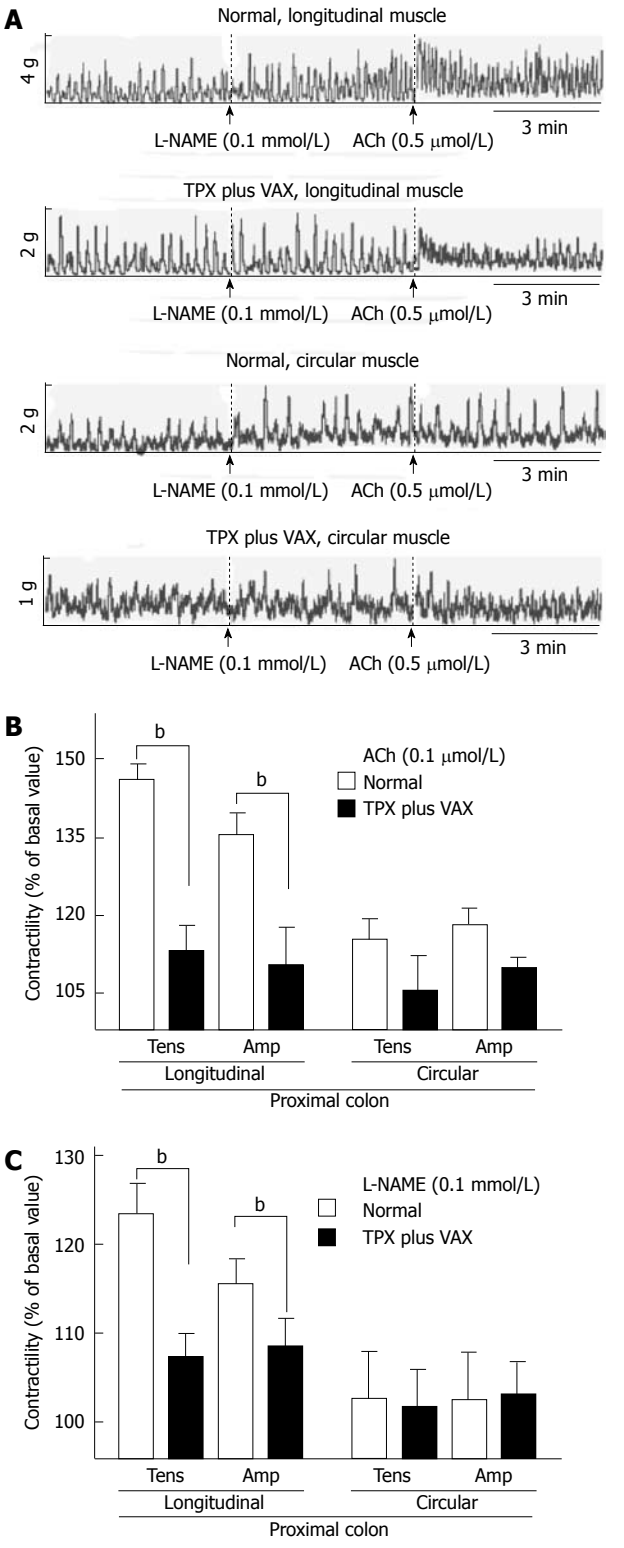

Figure 6 Reduced contractile activity of the proximal colonic muscles in the thyroparatyroidectomy plus subdiaphragmatic vagotomy rats. Animals were sacrificed at 8 d after subdiaphragmatic vagotomy (VAX), and intact proximal colonic strips prepared from the age-matched normal and thyroparatyroidectomy (TPX) plus VAX rats were longitudinally or circularly mounted in a 10 mL organ bath. The contractile responses of colonic strips to acetylcholine (Ach) and *N*-nitro-*L*-arginine methyl ester (L-NAME) were measured by isometric force transducers. Figures show the representative tracings (A) and contractile responses to ACh (B) and L-NAME (C). Each point represents the mean ± SE of 7-9 rats. $^{b}P < 0.01$ *vs* normal and sham-operated groups.

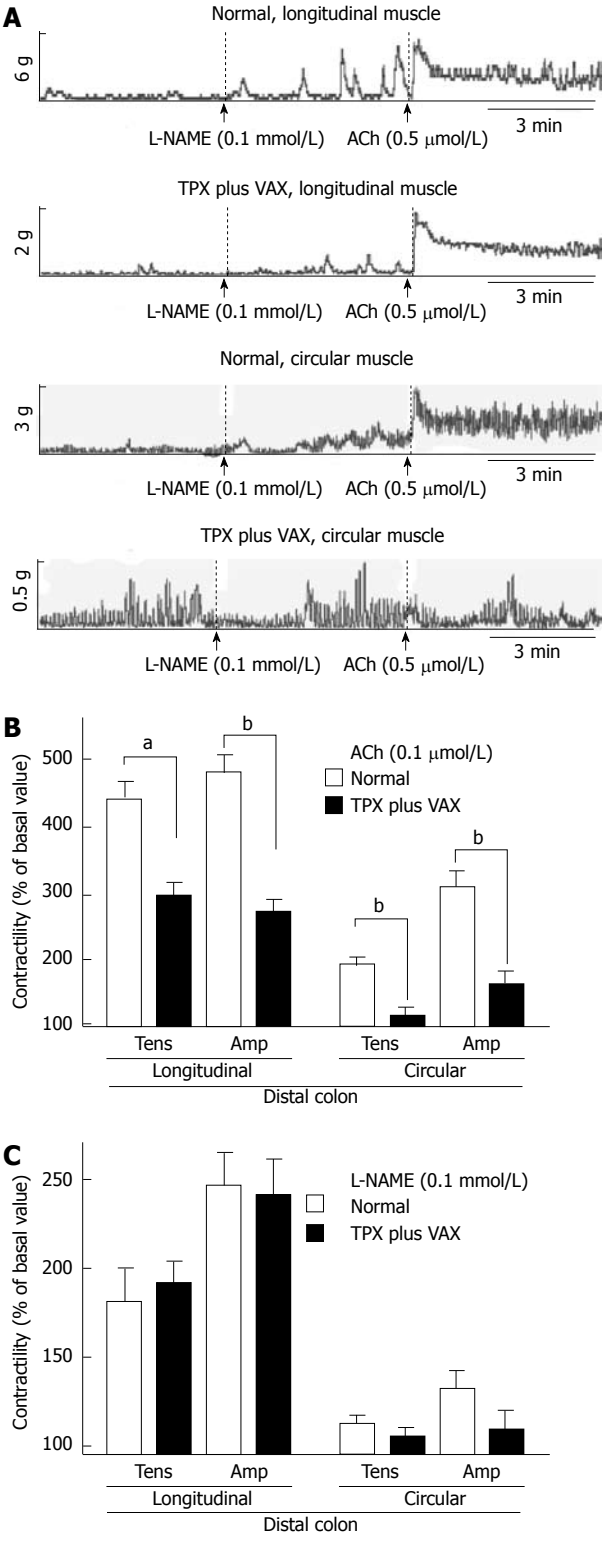

Figure 7 Reduced contractile activity of distal colonic muscles in the thyroparatyroidectomy plus subdiaphragmatic vagotomy rats. Animals were sacrificed at 8 d after subdiaphragmatic subdiaphragmatic vagotomy (VAX), and intact distal colonic strips prepared from the age-matched normal and thyroparatyroidectomy (TPX) plus VAX rats were longitudinally or circularly mounted in a 10 mL organ bath. The contractile responses of colonic strips to acetylcholine (Ach) and N-nitro-L-arginine methyl ester (L-NAME) were measured by isometric force transducers. Figures show the representative tracings (A) and contractile responses to ACh (B) and L-NAME (C). Each point represents the mean ± SE of 7-9 rats. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* normal and sham-operated groups.

DISCUSSION

To produce a rat model of a long-term reduction of the physiological motility of the entire GI tract, a partial depletion of thyroparathyroid hormones and denervation of subdiaphragmatic vagal nerves were sequentially applied to rats. In the rats receiving unilateral TPX plus VAX, an obvious dilatation of the stomach and significant residual luminal contents of GI tracts were found without marked morphological changes of the gut, and gastric emptying and small bowel transit were significantly delayed. The rats also showed a significant retardation of defecatory function. Additionally, we observed a significant reduction in distal colonic contractile responses to ACh, but not to L-NAME. These findings possibly indicate that a partial depletion of thyroparathyroid hormones and denervation of subdiaphragmatic vagal nerves decrease the spontaneous motility of the entire GI tracts through a reduction in the physiological activity of excitatory neurotransmitter systems. These dysfunctions are likely the pathological characteristics of the GI diseases related to reduced GI motility in humans.

Although a variety of animal models has been developed to examine the pathological mechanisms involved in the reduction of GI motility and to evaluate the pharmacological properties of drugs[19-22], it is unfortunately difficult to translate the findings of these animal models to human GI diseases. Although pathological characteristics of the bilateral TPX- or subdiaphragmatic VAX-induced animal models have been intensively investigated[1,3,6], the precise values for the combined method of unilateral TPX and subdiaphragmatic VAX are entirely, to our knowledge, lacking. Moreover, in the preliminary studies, we observed several scientific and methodological limitations of bilateral TPX or subdiaphragmatic VAX alone. A complete depletion of thyroparathyroid hormones by bilateral TPX caused an extremely high fatality rate (≥ 90%) within 48 h after surgery, and subdiaphragmatic VAX transiently (less than 3 d after surgery) reduced only gastric emptying and small bowel transit, but not colonic transit. These results led us to consider that bilateral TPX or subdiaphragmatic VAX may not be a reliable and reproducible method to produce animal models of GI diseases induced by long-term functional reduction of GI motility in humans.

It has been reported that subdiaphragmatic VAX in guinea pigs increased daily food intake and weight gain, whereas the reverse occurred in rats[23,24], and a pathological depletion of thyroid hormones causes a reduction in normal weight gain in rats[14]. These findings are partially in accord with our findings, indicating that unilateral TPX plus VAX significantly decreases body weight gain, but not daily dietary intake. We carefully consider that these may be related to the experimental methods used, which may differently regulate the physiological functions of gut including gastric secretion and bowel motility, resulting in different magnitudes of food intake and weight gain. This is supported by previous reports[1,25],

suggesting that alteration of food intake and weight gain following bilateral TPX and subdiaphragmatic VAX is absolutely dependent on the ability of the GI tract to digest food.

The delayed gastric emptying in some patients with hypothyroidism is likely to result from reduced muscle contractility of the antrum[26], and vagal nerves are involved in the regulation of contractile patterns during gastric phase Ⅲ[27]. In rats, chronic hypothyroidism or hyperthyroidism alters the expression patterns of cholinergic muscarinic receptors distributed in the ileal muscles[28], and administration of carbachol obviously increases ileal transit decline by vagal denervation[8]. These suggestions are well in accord with our results, indicating that unilateral TPX plus VAX markedly delays gastric emptying and small bowel transit. Although this study did not evaluate the cellular mechanisms implicated in the unilateral TPX plus VAX-induced reduction of upper GI motility, the results found in this study likely suggest that unilateral TPX plus VAX may be a reliable and reproducible method to produce a rat model characterized by a long-term reduction in gastric emptying and small bowel transit.

A previous report[8] suggested that colonic transit was delayed soon after subdiaphragmatic VAX, but returned to essentially normal levels at a later time. We also observed a similar effect of subdiaphragmatic VAX on defecatory function. Importantly, a reduction in defecatory function was maintained for 7 d when unilateral TPX and subdiaphragmatic VAX were sequentially applied to rats. These results clearly indicated that unilateral TPX plus VAX obviously reduces the colonic motor activity which propels the luminal contents, and the reduced propulsive activity of the upper and lower GI tract is maintained for long time periods compared with subdiaphragmatic VAX alone. Moreover, it is considered that a significant decrease in the daily numbers of fecal pellets is possibly caused by a reduction in the defecatory function of the colon, and this likely reflects the reduction in motor function of colonic smooth muscle observed in patients with constipation and defecatory disorders[25].

It has been well known that ACh[29] is the major excitatory neurotransmitter, and nitric oxide[30] is the major non-adrenergic, non-cholinergic inhibitory neurotransmitter in the regulation of colonic motility. Therefore, to assess the alteration in excitatory and inhibitory neurotransmitter systems by unilateral TPX plus VAX, we examined the contractile responses of colonic strips to ACh and L-NAME. In comparison with colonic strips of normal rats, the colonic strips prepared from unilateral TPX plus VAX rats exhibited a markedly impaired contractile response to ACh, but the contractile response to L-NAME was not significantly altered. We also found that unilateral TPX plus VAX did not significantly affect the contractile properties of adrenergic and purinergic inhibitory mechanisms (data not shown). These results are possibly suggestive of cholinergic neuronal remodeling during a partial depletion of thyroparathyroid hormones and disruption of subdiaphragmatic vagal nerves, resulting in functional reduction in colonic motility leading to pathological conditions such as constipation. Findings comparable with our results have previously been reported, suggesting that depletion of thyroparathyroid hormones reduces GI motility through a reduction in excitatory neurotransmitter activity[6]. Therefore, the results found in this study suggest that, in addition to dysfunction of vagal nerves regulating GI motility, the reduced colonic contractility may result from enteric excitatory neuronal loss associated with a decrease in thyroparathyroid hormones. However, further studies are needed to investigate the detailed cellular mechanisms involved in the unilateral TPX plus VAX-induced reduction of GI motility.

Taken together, sequential application of unilateral TPX and subdiaphragmatic VAX to rats may be a reliable method to produce a model of delayed gastric emptying and defecatory disorders in rats. In comparison with bilateral TPX or subdiaphragmatic VAX alone, TPX plus VAX showed valuable advantages, including no animal deaths and long-term persistence of reduced GI motility. Rats sequentially undergoing unilateral TPX and subdiaphragmatic VAX may be taken into consideration as a putative animal model for disorders of GI motility such as dyspepsia and constipation.

COMMENTS

Background

The prevalence of dyspepsia and constipation in humans continues to increase throughout the world. Although attenuation of gastric and colonic motility is widely considered as the primary causal factors for the occurrence of dyspepsia and constipation, respectively, there are still many questions to be answered, and a useful animal model to evaluate the pathophysiological mechanisms responsible for the reduction of gut motility remains to be developed.

Research frontiers

Thyroid diseases affect gastrointestinal (GI) functions including disturbance of gut motility, and the most common GI complaints in patients with hypothyroidism are nausea, vomiting, constipation and abdominal pain. The vagal nerves are extensively distributed in the digestive tracts from the upper esophageal sphincter muscles to the transverse colon in humans, and the extensive distribution of vagal nerves to the gut provides a key bi-directional link with the brain, which regulates GI behavior such as food intake, gastric accommodation and muscle contractility.

Innovations and breakthroughs

It has been demonstrated in our study using rats that complete depletion of thyroparathyroid hormones by thyroparathyroidectomy caused an extremely high fatality rate, and the disturbance of vagal activity by thyroparathyroidectomy produced a transient, less than 3 d, reduction of gastrointestinal motility. However, in comparison with thyroparathyroidectomy or vagotomy alone, application of both unilateral thyroparathyroidectomy and subdiaphragmatic vagotomy to rats had several advantages, including no deaths of the animals and a long-term, at least 7 d, reduction in gut motility. In the present study, the authors suggest for the first time that both unilateral thyroparathyroidectomy and vagotomy may be reliably applied to produce a rat model of GI disorders characterized by long-term reduction in GI motility.

Applications

Rats subject to both unilateral thyroparathyroidectomy and subdiaphragmatic thyroparathyroidectomy may provide an animal model of dyspepsia and constipation, and may be usefully used in studies of new drugs to reverse the reduced motility of the GI tract.

Terminology

Dyspepsia: A condition of impaired gastric digestive function associated with gastritis and gastroesophageal reflux. The common symptoms of dyspepsia are upper abdominal fullness, bloating, nausea, heartburn or feeling full earlier than expected.

Peer review

The authors have attempted to develop a novel animal model for reducing gastrointestinal motility in aid of studies examining dyspepsia and constipation. This is a straightforward study that addresses an important issue.

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BRIEF ARTICLE

Does immunohistochemical staining have a clinical impact in early gastric cancer conducted endoscopic submucosal dissection?

Seong Ran Jeon, Joo Young Cho, Gene Hyun Bok, Tae Hee Lee, Hyun Gun Kim, Won Young Cho, So Young Jin, Yeon Soo Kim

Seong Ran Jeon, Joo Young Cho, Gene Hyun Bok, Tae Hee Lee, Hyun Gun Kim, Won Young Cho, Institute for Digestive Research, Digestive Disease Center, Soonchunhyang University College of Medicine, Seoul 140-743, South Korea
So Young Jin, Department of Pathology, Soonchunhyang University College of Medicine, Seoul 140-743, South Korea
Yeon Soo Kim, Department of Internal Medicine, Daejeon St. Mary's Hospital, The Catholic University of Korea School of Medicine, Daejeon 301-723, South Korea
Author contributions: Jeon SR drafted the article, analysis and interpreted of the data; Bok GH, Lee TH, Kim HG, Cho WY, and Kim YS were responsible for analysis and interpretation of the data; Jin SY reviewed data analysis and corrected research design; Cho JY made the critical revision of the article for important intellectual content and final approval of the article.
Correspondence to: Joo Young Cho, Professor, MD, PhD, Institute for Digestive Research, Digestive Disease Center, Soonchunhyang University College of Medicine, 657 Hannam-Dong, Yongsan-Gu, Seoul 140-743, South Korea. cjy6695@dreamwiz.com
Telephone: + 82-2-7099202 Fax: +82-2-7099696
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Abstract

AIM: To evaluate clinicopathologic parameters and the clinical significance related lymphovascular invasion (LVI) by immunohistochemical staining (IHCS) in endoscopic submucosal dissection (ESD).

METHODS: Between May 2005 and May 2010, a total of 348 lesions from 321 patients (mean age 63 ± 10 years, men 74.6%) with early gastric cancer (EGC) who met indication criteria after ESD were analyzed retrospectively. The 348 lesions were divided into the absolute (n = 100, differentiated mucosal cancer without ulcer ≤ 20 mm) and expanded (n = 248) indication groups after ESD. The 248 lesions were divided into four subgroups according to the expanded ESD indication. The presence of LVI was determined by factor Ⅷ-related antigen and D2-40 assessment. We compared LVI IHCS-negative group with LVI IHCS-positive in each group.

RESULTS: LVI by hematoxylin-eosin staining (HES) and IHCS were all negative in the absolute group, while was observed in only the expanded groups. The positive rate of LVI by IHCS was higher than that of LVI by HES (n = 1, 0.4% *vs* n = 11, 4.4%, P = 0.044). LVI IHCS-positivity was observed when the cancer invaded to the mucosa 3 (M3) or submucosa 1 (SM1) levels, with a predominance of 63.6% in the subgroup that included only SM1 cancer (P < 0.01). In a univariate analysis, M3 or SM1 invasion by the tumor was significantly associated with a higher rate of LVI by IHCS, but no factor was significant in a multivariate analysis. There were no cases of tumor recurrence or metastasis during the median 26 mo follow-up.

CONCLUSION: EGCs of the absolute group are immunohistochemically stable. The presence of LVI may be carefully examined by IHCS in an ESD expanded indication group with an invasion depth of M3 or greater.

Key words: Gastric cancer; Endoscopic submucosal dissection; Immunohistochemical staining; Lymphovascular invasion; Depth

Peer reviewers: Magdy El-Salhy, Professor, Department of Gastroenterology, School of Medicine, Stord Helse-Fonna Hospital, 5409 Stord, Norway; Dr. Cuneyt Kayaalp, Professor, Depatment of General Surgery, Inonu University, 44315 Malatya, Turkey

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INTRODUCTION

The incidence of early gastric cancer (EGC) has increased in Asia, and particularly in South Korea and Japan[1]. Endoscopic submucosal dissection (ESD) can be used for *en bloc* and complete resection (CR), and is widely applied for the curative treatment of EGC[2-4]. Studies comparing the long-term results of EGC treatment have demonstrated no significant increase in the five-year survival rate of patients following ESD[2,5,6]. However, these techniques are necessary for proper diagnosis of tumor histology to check whether the tumor has been completely removed or has recurred and to predict the possibility of lymph node (LN) metastasis, thereby determining the need for additional surgery.

Assessment of lymphovascular invasion (LVI) is usually performed on the basis of conventional hematoxylin-eosin staining (HES), sometimes lacks objectivity possibly because of the inability to distinguish lymphatics from blood vessels[7]. Although the clinical significance of molecular-based methods using immunohistochemical staining (IHCS) of resected tissues is controversial, IHCS has been used to evaluate the presence of LVI, which a risk factor for LN metastasis[8-10]. Thus, we evaluated possible correlations between IHCS and other clinicopathologic parameters in ESD specimens and the clinical significance of IHCS.

MATERIALS AND METHODS

Study design and patients

Data were retrospectively collected from patients who underwent ESD at a single, tertiary-care, academic medical center. Between May 2005 and May 2010, a total of 348 lesions (321 patients) that met the absolute and expanded indication criteria after ESD were included. This study was approved by our hospital ethics committee and institutional review board. Written informed consent was obtained from all patients prior to the procedure.

Pre-ESD evaluation

The pre-ESD assessment of tumor size and depth of invasion was performed by conventional endoscopy and endoscopic ultrasonography. Contrast-enhanced computed tomography was also performed in all patients before treatment to exclude involvement of lymph nodes or distant metastasis.

ESD and pathologic analysis

ESD was performed as reported previously[11,12]. All ESD specimens were examined by an experienced pathologist (Jin SY). Resected specimens were fixed in 10% formalin and sectioned serially in 2-mm intervals, then subjected to histological analysis. All slices were embedded in a paraffin block. The section margin, depth of invasion, and LVI were observed carefully. A one-piece resection was defined as an *en bloc* resection. The definition of CR was: (1) *en bloc* resection or complete reconstruction in two piecemeal resection cases; (2) being free of cancer at both the vertical (VM) and lateral margins (LM); (3) no submucosal invasion deeper than 500 μm from the muscularis mucosa; and (4) no evidence of vascular or lymphatic invasion. Incomplete resection was defined as resections that did not meet the curative criteria. Incompletely resected lesions confined to the mucosa with positive LM were scheduled for an additional ESD procedure, argon plasma coagulation, or surgery.

In pathology specimens obtained by ESD, the absolute indication criteria for ESD was intramucosal differentiated adenocarcinoma without ulceration $\leqslant$ 20 mm in diameter. The expanded indication criteria for ESD were: (1) intramucosal differentiated adenocarcinoma without ulceration > 20 mm in diameter; (2) intramucosal differentiated adenocarcinoma with ulceration $\leqslant$ 30 mm in diameter; (3) submucosa 1 (SM1) differentiated adenocarcinoma $\leqslant$ 30 mm in diameter; and (4) intramucosal undifferentiated adenocarcinoma without ulceration $\leqslant$ 20 mm in diameter. In this order, the expanded indication group was divided into four subgroups (groups 1, 2, 3 and 4).

Immunohistochemical staining

Serial sections (4 μm-thick) were deparaffinized in xylene. Samples were rehydrated in a graded alcohol series, and heated for 10 min in a microwave oven. This procedure was repeated twice after immersion in a citrate buffer solution to increase the reproducibility of endogenous antigens. To detect endogenous peroxidase, the samples were placed in 3% hydrogen peroxide solution for 10 min and rinsed with phosphate-buffered saline (PBS). Nonspecific binding sites were blocked by incubation in 3% normal non-immune serum for 10 min. Then the slides were washed with PBS and incubated with biotinylated polyvalent secondary antibody at room temperature for 15 min. After washing with PBS, the slides were incubated with streptavidine peroxidase for 15 min and washed a second time. Immunoreactive proteins were visualized by incubation with amine-ethyl carbazole for 3 to 5 min. The samples were counterstained with Mayer's HE and mounted using crystal mount (Biomeda, Foster City, CA, United States).

Primary antibodies specific for the mucin and gastric phenotypic markers, MUC5AC (CLH2, Novocastra Lab. Ltd, Newcastle, United Kingdom) and MUC6 (CLH5, Novocastra Lab. Ltd, Newcastle, United Kingdom), the intestinal phenotypic marker MUC2 (CCP58, Biogenx, San Ramon, CA, United States), and CD10 (56C6, Signet Lab. Oakland, CA, United States) were used. Samples that were positive for MUC5AC or MUC6 were classi-

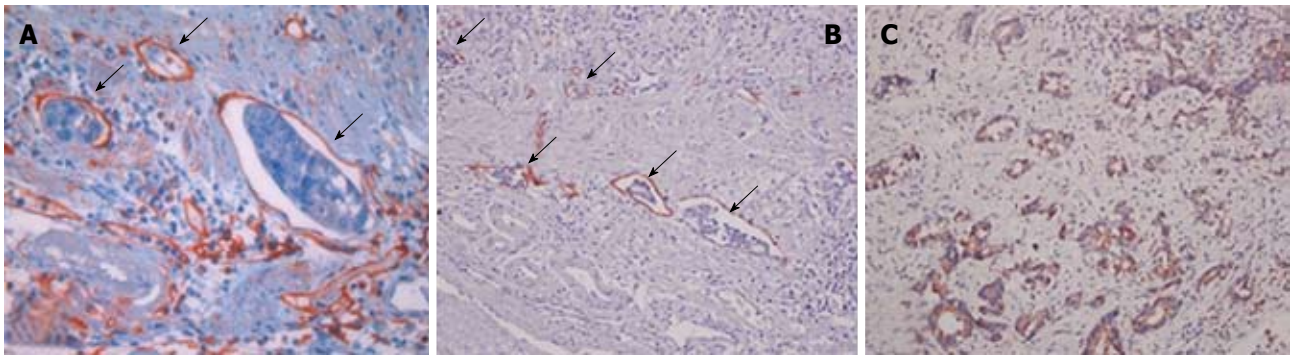

Figure 1 Immunohistochemical findings of early gastric cancers. A: Three foci of vascular invasion of tumor cells (arrows) are seen (factor Ⅷ related antigen, × 400); B: Multiple foci of lymphatic invasion (arrows) are found in the both mucosa and submucosa (D2-40, ×200); C: Positive staining is noted in the cytoplasm of neoplastic glands (vascular endothelial growth factor, ×200).

fied as having a gastric phenotype, and those positive for MUC2 or CD10 were classified as having an intestinal phenotype. Samples that were positive for at least one marker of both phenotypes were considered to have a mixed phenotype, and samples showing no marker immunoreactivity were considered to be an unclassified phenotype.

Lymphovascular-related markers and IHCS analysis

For the vascular endothelial marker, primary antibodies against factor Ⅷ-related antigen (FⅧRAg, 1:3000; DAKO, Carpenteria, CA, United States) were used. For the lymphatic endothelial marker, primary antibodies against D2-40 (1:200; LI-D, Covance Inc., Cumberland, VA, United States) were used. Primary antibodies against vascular endothelial growth factor (VEGF) (1:50; SC-152, Santa Cruz Biotechnology, Santa Cruz, CA, United States) were also applied. Vascular or LVI was indicated by clusters of tumor cell in vessels or lymphatic vasculature, the endothelia of which stained positive for FⅧRAg or D2-40, respectively. VEGF was considered positive when anti-VEGF antibodies stained the tumor cytoplasm. High microvessel density (MVD) and lymphovascular density (LVD) were detected by increased intra-tumor staining of factor Ⅷ and D2-40 (Figure 1).

Post-ESD follow-up

Endoscopic follow-up examinations were performed routinely at 1, 3, 6 and 12 mo, and then annually after ESD to assess the completeness of resection and to detect metachronous lesions. During the follow-up period, biopsy samples were taken from the ESD ulcer scar or other suspicious mucosal abnormalities. If a residual lesion or metachronous lesion was noted, additional ESD or surgery was planned.

Statistical analysis

Statistical analyses were performed using the SPSS software (version 15.0; SPSS, Chicago, IL, United States). Categorical variables were analyzed by the Chi-square test or Fisher's exact test and non-categorical variables were analyzed by the *t* test to verify the relationship between IHCS and other clinicopathological parameters. Non-categorical variables were compared among the four groups using an analysis of variance test. A multivariate analysis was conducted using a logistic regression method. *P* values $\leqslant$ 0.05 indicated statistical significance.

RESULTS

Endoscopic and pathologic characteristics

In the absolute group, the mean age of the 100 patients (100 lesions) was 62.2 years (range: 33-86 years) and included 70 men (70%). In the expanded group, the mean age of the 221 patients (248 lesions) was 62.9 years (range: 33-91 years) and included 189 men (76.2%). The dominant tumor location in both groups was the lower third of the stomach. Macroscopically, the flat/depressed type was most common (n = 131, 52.8%), and ulcers were observed in 64.9% (n = 161) of patients in the expanded group.

Of 248 lesions in the expanded group, 238 (96%) were differentiated and 10 (4%) were poorly differentiated adenocarcinoma. Twenty-eight lesions (11.3%) were SM1 cancer. The mean tumor size was 21.2 mm (range: 3-86 mm). Twenty-four (9.7%) were LM-positive two (0.8%) were VM-positive. The mixed mucin phenotype (n = 90, 36.3%) was predominant. A total of 72 (29%) were VEGF positive and 59 (23.8%) exhibited a high MVD/LVD. The CR rate by IHCS was significantly lower than that by HES (84.3% *vs* 88.3%, $P < 0.01$). The endoscopic and histopathologic characteristics of the lesions in the absolute and expanded groups are summarized in Table 1.

Subgroup analysis in the expanded indication

As mentioned above, the 248 lesions were divided into four groups according to the expanded ESD indication. Groups 1, 2, 3 and 4 contained 71 (28.6%), 140 (56.4%), 27 (10.8%) and 10 (4.7%) lesions, respectively. LVI HES-positivity was also defined as when LVI was positive by HE staining, and LVI IHCS-positivity was also defined as when LVI was positive by IHCS. Of a number of clinicopathological factors, only LVI IHCS-positivity showed significant differences between subgroups. LVI

Table 1 Endoscopic and pathologic characteristics of endoscopic submucosal dissection groups

Groups	Absolute (n = 100)	Expanded (n = 248)	P value
Age (yr), mean (range)	62.2 (33-86)	62.9 (33-91)	0.589
Male (%)	70 (70)	189 (76.2)	0.277
Location (%)			0.183
Upper	3 (3)	21 (8.5)	
Middle	31 (31)	69 (27.8)	
Lower	66 (66)	158 (63.7)	
Macroscopic type (%)			< 0.001
Elevated	59 (59)	56 (22.6)	
Flat/depressed	36 (36)	131 (52.8)	
Mixed	5 (5)	61 (24.6)	
Endoscopic ulcer (%)			< 0.001
Present	0 (0)	161 (64.9)	
Absent	100 (100)	87 (35.1)	
Resection (%)			0.053
En-bloc	100 (100)	234 (94.4)	
Complete reconstruction	0 (0)	8 (3.2)	
⩾ 3 piecemeal	0 (0)	6 (2.4)	
Histology (%)			0.069
Differentiated	100 (100)	238 (96)	
Undifferentiated	0 (0)	10 (4)	
Depth (%)			< 0.001
M2	59 (59)	89 (35.9)	
M3	41 (41)	131 (52.8)	
SM1	0 (0)	28 (11.3)	
Tumor size (mean, range)	11.4 (2-20)	21.2 (3-86)	< 0.001
⩽ 20 mm (%)	100 (100)	131 (52.8)	
> 20 mm (%)	0 (0)	117 (47.2)	
Lateral margin (%)			< 0.001
Positive	0 (0)	24 (9.7)	
Negative	100 (100)	224 (90.3)	
Vertical margin (%)			0.500
Positive	0 (0)	2 (0.8)	
Negative	100 (100)	246 (99.2)	
Phenotype (%)			0.312
Gastric	24 (24)	66 (26.6)	
Intestinal	31 (31)	92 (37)	
Mixed	45 (45)	90 (36.3)	
VEGF (%)			0.096
Positive	79 (79)	72 (29)	
Negative	21 (21)	176 (71)	
High MVD/LVD (%)			0.116
Positive	16 (16)	59 (23.8)	
Negative	84 (84)	189 (76.2)	
LVI HES			0.713
Positive	0 (0)	1 (0.4)	
Negative	100 (100)	247 (99.6)	
LVI IHCS			0.038
Positive	0 (0)	11 (4.4)	
Negative	100 (100)	237 (95.6)	

M: Mucosa; SM: Submucosa; VEGF: Vascular endothelial growth factor; MVD: Microvessel density; LVD: Lymphatic vessel density; LVI: Lymphovascular invasion; HES: Hematoxylin-eosin staining; IHCS: Immunohistochemical staining.

HES-positivity was observed only in group 4, which included undifferentiated carcinoma. While LVI IHCS-positivity was observed in all subgroups, there was a predominance of 63.6% (7/11) in group 3, which included only SM1 cancer ($P < 0.001$) (Table 2).

Comparison of LVI in the immunohistochemically negative and positive groups in the expanded indication

LVI HES-positivity was confirmed by LVI IHCS; however, of 11 lesions identified as LVI IHCS-positive, only one was also LVI HES-positive (100% *vs* 9.1%, $P = 0.044$) (Table 3).

The association between LVI IHCS and various clinicopathological factors and IHCS markers was analyzed (Table 4). In this statistical analysis, we combined the three tumor location and depth of invasion categories into two "upper + corpus" and "lower + angle", and "mucosa 2 (M2)" and "M3 + SM1", respectively. Phenotype was categorized into intestinal and non-intestinal type. LVI IHCS-positivity was detected in 4.4% (11/248). In a univariate analysis, M3 or SM1 invasion of the tumor, and the presence of VEGF and a high LVD/MVD were factors significantly associated with a higher rate of LVI by IHCS. However, there were no significant as-

Table 2 Subgroup analysis in the expanded indication groups

	Group 1 (n = 71)	Group 2 (n = 140)	Group 3 (n = 27)	Group 4 (n = 10)	P value
Age > 60 (yr, %)	44 (62)	82 (58.6)	21 (77.8)	6 (60)	0.502
Male (%)	56 (78.9)	104 (74.3)	24 (88.9)	5 (50)	0.455
Location (%)					0.730
Upper + corpus	19 (26.8)	15 (10.7)	11 (40.7)	1 (10)	
Lower + angle	52 (73.2)	125 (89.3)	16 (59.3)	9 (90)	
Macroscopic type (%)					< 0.001
Elevated	45 (63.4)	4 (2.9)	4 (14.8)	3 (30)	
Flat/depressed	26 (36.6)	136 (97.1)	23 (85.2)	7 (70)	
Endoscopic ulcer (%)					< 0.001
Present	0 (0)	140 (100)	20 (74.1)	1 (10)	
Absent	71 (100)	0 (0)	7 (25.9)	9 (90)	
Resection, piece (%)					0.177
⩽ 2	68 (95.8)	137 (97.9)	27 (100)	10 (100)	
⩾ 3	3 (4.2)	3 (2.1)	0 (0)	0 (0)	
Depth (%)					0.003
M2	33 (46.5)	52 (37.1)	0 (0)	4 (40)	
M3 + SM1	38 (53.5)	88 (62.9)	27 (100)	6 (60)	
Tumor size, mm (%)					< 0.001
⩽ 20	0 (0)	105 (75)	16 (59.3)	10 (100)	
> 20	71(100)	35 (25)	11 (40.7)	0 (0)	
Lateral margin (%)					0.091
Positive	10 (14.1)	11 (7.9)	1 (3.7)	2 (20)	
Negative	61 (85.9)	129 (92.1)	26 (96.3)	8 (80)	
Vertical margin (%)					0.170
Positive	2 (2.8)	0 (0)	0 (0)	0 (0)	
Negative	69 (97.2)	140 (100)	27 (100)	10 (100)	
Phenotype (%)					0.107
Intestinal	31 (43.7)	50 (35.7)	9 (33.3)	2 (20)	
Non-intestinal	40 (56.3)	90 (64.3)	18 (66.7)	8 (80)	
VEGF (%)					0.003
Positive	17 (23.9)	34 (24.3)	16 (59.3)	5 (50)	
Negative	54 (76.1)	106 (75.7)	11 (40.7)	5 (50)	
High LVD/MVD (%)					< 0.001
Positive	11 (15.5)	26 (18.6)	16 (59.3)	6 (60)	
Negative	60 (84.5)	114 (81.4)	11 (40.7)	4 (40)	
LVI HES					0.005
Positive	0 (0)	0 (0)	0 (0)	1 (10)	
Negative	71 (100)	140 (100)	27 (100)	9 (90)	
LVI IHCS					< 0.001
Positive	1 (1.4)	1 (0.7)	7 (25.9)	2 (20)	
Negative	70 (98.6)	139 (99.3)	20 (74.1)	8 (80)	

M: Mucosa; SM: Submucosa; VEGF: Vascular endothelial growth factor; MVD: Microvessel density; LVD: Lymphatic vessel density; LVI: Lymphovascular invasion; HES: Hematoxylin-eosin staining; IHCS: Immunohistochemical staining.

Table 3 Comparison of lymphovascular invasion hematoxylin-eosin staining and lymphovascular invasion immunohistochemical staining groups

ESD indication			LVI IHCS		Total	*P* value
			Negative	Positive		
Absolute	LVI HES	Negative	100	0	100	
		Positive	0	0	0	
	Total		100	0	100	
Expanded	LVI HES	Negative	237	10	247	0.044
		Positive	0	1	1 (0.4%)	
	Total		237	11 (4.4%)	248	

ESD: Endoscopic submucosal dissection; LVI: Lymphovascular invasion; HES: Hematoxylin-eosin staining; IHCS: Immunohistochemical staining.

sociations between LVI by IHCS and age, sex, location, macroscopic type, endoscopic ulcer, resection pieces, histology, tumor size, lateral margin, vertical margin, or phenotype. In a multivariate analysis, there were no significant associations between LVI in IHCS and these factors (Table 4).

Follow-up

Of the 321 patients who underwent ESD, 5 (1.6%) required additional surgery (Table 5). The reasons for additional surgery were an insufficient gap between the tumor and LM or VM in four patients and patient need in one. No lesions exhibited LN metastasis. Two of the five patients had a residual tumor in the gastric wall. There were no cases of tumor recurrence or metastasis after ESD, including those patients who underwent additional surgery, during the median 26 mo follow-up.

DISCUSSION

LVI and increased LVD detected by IHCS are associated with LN metastasis in gastric cancer[13-18], and are correlated with an unfavorable prognosis in other types of cancer[19-23]. However, detection of LVI by HES is difficult for several reasons. Primarily, the distinction between blood vessels and lymphatic vessels is subjective. Furthermore, artifacts or artificial spaces formed during sample preparation may mimic the tubular structure of vasculature[7]. Antibodies to FⅧRAg help to distinguish true vascular endothelial cells therefore increasing the sensitivity of detection of vascular invasion[24,25] whereas the monoclonal antibody D2-40 specific for lymphatic endothelial cells enhances detection of lymphatic invasion[26-28]. In a previous study using post-operative specimens to diagnose lymphatic invasion, D2-40 was more sensitive for detection of lymphatic invasion (positive rate 44% *vs* 27% for D2-40 *vs* HE)[27]. Thus, we evaluated the clinical significance of IHCS, using for example FⅧRAg and D2-40, in patients with the expanded indication criteria after ESD. To the best of our knowledge, no study has evaluated the possible correlations between IHCS and other clinicopathological parameters in the expanded indication of ESD.

Table 4 Comparison of lymphovascular invasion by immunohistochemical stain negative and positive group

	LVI IHCS		*P* value
	Negative (*n* = 237)	Positive (*n* = 11)	
Age, yr (%)	62.6 ± 9.9	69.4 ± 10.2	0.054
≤ 60	92 (38.8)	3 (27.3)	
> 60	145 (61.2)	8 (72.7)	
Male (%)	182 (76.8)	7 (63.6)	0.297
Location (%)			0.667
Upper + corpus	44 (18.6)	2 (18.2)	
Lower + angle	193 (81.4)	9 (81.8)	
Macroscopic type (%)			0.715
Elevated	53 (22.4)	3 (27.3)	
Flat/depressed	184 (77.6)	8 (72.7)	
Endoscopic ulcer (%)			0.524
Present	155 (65.4)	6 (54.5)	
Absent	82 (34.6)	5 (45.5)	
Resection, piece (%)			0.760
≤ 2	231 (97.5)	11 (100)	
≥ 3	6 (2.5)	0 (0.0)	
Histology (%)			0.066
Differentiated	229 (96.6)	9 (81.8)	
Undifferentiated	8 (3.4)	2 (18.2)	
Depth (%)			0.009
M2	89 (37.6)	0 (0)	
M3 + SM1	148 (62.4)	11 (100)	
Tumor size, mm (%)	21.2 ± 12.8	21.2 ± 16.7	0.986
≤ 20	125 (52.7)	6 (54.5)	
> 20	112 (47.3)	5 (45.5)	
Lateral margin (%)			0.289
Positive	22 (9.3)	2 (18.2)	
Negative	215 (90.7)	9 (81.8)	
Vertical margin (%)			0.913
Positive	2 (0.8)	0 (0)	
Negative	235 (99.2)	11 (100)	
Phenotype (%)			0.751
Intestinal	89 (37.6)	3 (27.3)	
Non-intestinal	148 (62.4)	8 (72.7)	
VEGF (%)			< 0.001
Positive	62 (26.2)	10 (90.9)	
Negative	175 (73.8)	1 (9.1)	
High LVD/MVD (%)			< 0.001
Positive	48 (20.3)	11 (100)	
Negative	189 (79.7)	0 (0.0)	

IHCS: Immunohistochemical staining; M: Mucosa; SM: Submucosa; VEGF: Vascular endothelial growth factor; LVD: Lymphatic vessel density, MVD: Microvessel density.

In this study, 11 lesions in the only expanded indication group, but not the absolute indication group, displayed LVI-positivity. This is likely because lymphatics existed only in the deep mucosa and abundant microvessels were found in the muscularis mucosa[27]. Differentiated mucosal cancer, which belongs to the absolute indication group was LVI-negative. Because the EGCs of the absolute indication group were immunohistochemically stable, the clinical implication of IHCS in this group is insignificant.

In the expanded indication, comparing the LVI IHCS-negative and LVI IHCS-positive groups, M3 or SM1 invasion of the tumor and the presence of VEGF and a high LVD/MVD were factors significantly associated with a higher rate of LVI by IHCS. However,

Table 5 Clinicopathological characteristics of the five patients who underwent surgical resection after endoscopic submucosal dissection

Sex/age (yr)	Type	Site	Ulcer	Pathology	Size (mm)	LM/VM	Depth	LVI HES	LVI IHCS	Residual tumor	LN
Female/67	Ⅱc	Lower	-	MD	30	-/+	M	-	-	+	-
Male/74	Ⅲ	Middle	+	WD	14	+/-	M	+	+	-	-
Female/64	Ⅲ	Middle	+	MD	19	+/-	M	-	-	-	-
Female/54	Ⅰ	Middle	-	MD	68	+/-	M	-	+	+	-
Male/35	Ⅰ	Middle	-	MD	22	-/-	M	-	-	-	-

LM: Lateral margin; VM: Vertical margin; LVI: Lymphovascular invasion; HES: Hematoxylin-eosin staining; IHCS: Immunohistochemical staining; LN: Lymph node; MD: Moderately differentiated; WD: Well differentiated; M: Mucosa.

there were no significant associations between LVI by IHCS and these factors in a multivariate analysis. This may be because, first, the number of patients in the LVI IHCS-positive group was too small compared to the LVI IHCS-negative group. Second, although the specimens were examined by a highly experienced pathologist, the interpretation of the IHCS results was not always accurate. Nonetheless, we must not overlook the fact that LVI-positivity was observed when the cancer invaded the M3 or SM1 layer in the LVI IHCS-positive group. Resected specimens examined by HES showed an LVI false-negative rate of 4% and a false-positive rate of 0%. The positive rate of LVI by IHCS was significantly higher than that of LVI by HES (4% *vs* 0.4%, $P = 0.044$). In other words, 10 tumors, which had been diagnosed as no LVI by HES, were correctly diagnosed as having LVI after IHCS. Only 1 of 11 (9%) LVI, confirmed by IHCS, was correctly diagnosed by HES. These results suggest that LVI cannot be predicted by HES alone and also that IHCS is somewhat more helpful than HES for identifying LVI. Therefore, the presence of LVI should be carefully examined by IHCS when invasion with a depth of M3 or greater has been detected in an expanded indication.

The major limitations of this study are its retrospective design, the relatively short follow-up period and the limited number of patients with LVI by IHCS. Because the number of LVI by IHCS was small and additional surgical treatment was not provided to all patients who were LVI-positivity by IHCS, we were unable to explain the association between LVI IHCS-positivity and LN metastasis. According to the Japanese classification of gastric carcinoma[29], resected specimens were cut thinner (*vs* surgical specimens; 2 mm *vs* 4 mm). Therefore, the quality of pathologic interpretation of resected specimens was better than that of surgical specimens. We found no case of metastasis in the LVI IHCS-positive group during the median 26 mo follow-up. However, because a previous study reported that LN metastasis was confirmed in 89% of D2-40 positive cases, compared to 41% of suspected cases of metastasis based on HES[14], a future large and long-term follow-up study is needed to assess via IHCS the relationship of LVI with LN metastasis, tumor recurrence, and patient survival.

Examination by IHCS may be required because LVI can be undetected in an ESD expanded indication group with an invasion depth of M3 or greater if we rely only on HES.

COMMENTS

Background

Lymphovascular invasion (LVI) detected by immunohistochemical staining (IHCS) is associated with lymph node (LN) metastasis in gastric cancer. The clinical significance of IHCS in early gastric cancer (EGC) in association with endoscopic submucosal dissection (ESD) is controversial. No study has evaluated the possible correlations between IHCS and other clinicopathological parameters in ESD specimens.

Research frontiers

Relationships between LN micrometastasis and clinicopathological findings based on D2-40 and factor Ⅷ-related antigen (FⅧRA) IHCS in gastric cancer have been reported. Several studies have reported that IHCS is more sensitive for detection of LVI.

Innovations and breakthroughs

LVI by hematoxylin-eosin stain (HES) and IHCS were all negative in an ESD absolute group; this group was immunohistochemically stable. However, in an ESD expanded group, the LVI IHCS-positive rate was significantly higher than that of LVI by HES. LVI IHCS-positivity was observed when the cancer invaded the mucosa 3 (M3) or submucosa 1 (SM1) levels. Therefore, the presence of LVI should be carefully examined by IHCS in an expanded group with an invasion depth of M3 or SM1.

Applications

In an ESD expanded indication group with an invasion depth of M3 or greater, IHCS should be used because LVI-positivity can remain undetected by HES. However, a large-scale prospective study is required.

Terminology

ESD, a method that can be used for *en bloc* and complete resection of EGC, is widely applied for the curative treatment of EGC. IHCS is widely used in the diagnosis of abnormal cells using specific molecular markers, such as D2-40 and FⅧRA.

Peer review

Because the number of LVIs detected by IHCS was small and additional surgical treatment was not provided to all patients who were LVI-positive by IHCS, it was unable to explain the association between LVI IHCS-positivity and LN metastasis. However, this study implies the clinical significance of IHCS in an ESD expanded indication group, and reports a useful methodology.

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BRIEF ARTICLE

Spectral analysis of bowel sounds in intestinal obstruction using an electronic stethoscope

Siok Siong Ching, Yih Kai Tan

Siok Siong Ching, Yih Kai Tan, Department of Surgery, Changi General Hospital, Singapore 529889, Singapore
Author contributions: Ching SS conceived and designed the study, provided supporting material, acquired the data, performed the statistical analysis, interpreted the data and wrote the manuscript; Tan YK supervised the study and critically revised the manuscript.
Correspondence to: Siok Siong Ching, MRCS, MD, Department of Surgery, Changi General Hospital, 2 Simei Street 3, Singapore 529889, Singapore. ching_ss@hotmail.com
Telephone: +65-97729648 Fax: +65-62601709
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Abstract

AIM: To determine the value of bowel sounds analysis using an electronic stethoscope to support a clinical diagnosis of intestinal obstruction.

METHODS: Subjects were patients who presented with a diagnosis of possible intestinal obstruction based on symptoms, signs, and radiological findings. A 3M™ Littmann® Model 4100 electronic stethoscope was used in this study. With the patients lying supine, six 8-second recordings of bowel sounds were taken from each patient from the lower abdomen. The recordings were analysed for sound duration, sound-to-sound interval, dominant frequency, and peak frequency. Clinical and radiological data were reviewed and the patients were classified as having either acute, subacute, or no bowel obstruction. Comparison of bowel sound characteristics was made between these subgroups of patients. In the presence of an obstruction, the site of obstruction was identified and bowel calibre was also measured to correlate with bowel sounds.

RESULTS: A total of 71 patients were studied during the period July 2009 to January 2011. Forty patients had acute bowel obstruction (27 small bowel obstruction and 13 large bowel obstruction), 11 had subacute bowel obstruction (eight in the small bowel and three in large bowel) and 20 had no bowel obstruction (diagnoses of other conditions were made). Twenty-five patients received surgical intervention (35.2%) during the same admission for acute abdominal conditions. A total of 426 recordings were made and 420 recordings were used for analysis. There was no significant difference in sound-to-sound interval, dominant frequency, and peak frequency among patients with acute bowel obstruction, subacute bowel obstruction, and no bowel obstruction. In acute large bowel obstruction, the sound duration was significantly longer (median 0.81 s *vs* 0.55 s, $P = 0.021$) and the dominant frequency was significantly higher (median 440 Hz *vs* 288 Hz, $P = 0.003$) when compared to acute small bowel obstruction. No significant difference was seen between acute large bowel obstruction and large bowel pseudo-obstruction. For patients with small bowel obstruction, the sound-to-sound interval was significantly longer in those who subsequently underwent surgery compared with those treated non-operatively (median 1.29 s *vs* 0.63 s, $P < 0.001$). There was no correlation between bowel calibre and bowel sound characteristics in both acute small bowel obstruction and acute large bowel obstruction.

CONCLUSION: Auscultation of bowel sounds is non-specific for diagnosing bowel obstruction. Differences in sound characteristics between large bowel and small bowel obstruction may help determine the likely site of obstruction.

Key words: Bowel sounds; Intestinal obstruction; Spectral analysis; Electronic stethoscope

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INTRODUCTION

Auscultation of bowel sounds is a traditional technique for evaluating patients with abdominal symptoms. It is simple, but generally empirical and too subjective. Bowel sounds show wide variations from person to person and even from the same person at different times. The interpretation of a person's bowel sounds by different clinicians may also vary[1,2]. There is a distinct lack of clinical research within the literature to support any discussion on the value of auscultation for bowel sounds[3]. Many practitioners pay little attention to this physical examination and some have even abandoned their stethoscope. The question has been raised as to whether auscultation for bowel sounds is of any clinical value.

Recently, recording of bowel sounds with objective evaluation has become possible with commercially available electronic stethoscopes; however, bowel sounds in intestinal obstruction have not been extensively studied since the introduction of these stethoscopes.

The aim of this study is to determine the value of objective assessment of bowel sounds provided by an electronic stethoscope in supporting a clinical diagnosis of intestinal obstruction. Correlation with radiological and operative findings and clinical outcome is made to identify characteristic bowel sounds that will improve the diagnostic accuracy for bowel obstruction.

MATERIALS AND METHODS

The electronic stethoscope used for this study was the 3M™ Littmann® Model 4100 with Version 2.0 sound analysis software (3M Health Care, St. Paul, MN 55144, United States). Three frequency modes are available for auscultation: bell (20-200 Hz), diaphragm (100-500 Hz), and extended range (20-1000 Hz). Bowel sounds are best heard in the diaphragm mode, which selectively filters in sounds of higher frequency range, thus omitting the low frequency sounds transmitted from the heart or lungs. Other features of the electronic stethoscope include the ability to amplify sounds several times compared with a conventional stethoscope, and digital signal processing over the entire acoustic range. A maximum of six distinct tracks of up to 8 s each can be recorded and stored in the stethoscope. The recorded sound tracks are then transmitted to a computer via an infrared link.

The Local Ethics Committee approved this study. The subjects were patients admitted to our institution with a diagnosis of possible intestinal obstruction during the period July 2009 to January 2011. Clinical data were prospectively collected. The presenting complaints and findings from physical examination were reviewed following admission to the ward. Verbal consent was obtained from the patients for recording of their bowel sounds. The patients were positioned lying supine on a bed. Six 8-s recordings were taken from each patient with the stethoscope positioned on the lower abdomen (two at the right iliac fossa, two at the suprapubic region, and two at the left iliac fossa). The reason for recording sounds from the three different areas of the lower abdomen was to position the stethoscope as far away from the heart and lungs as possible to reduce the interference by sounds arising from the heart and lungs. For the purpose of this study, no attempt was made to compare the sounds at different recording sites as a previous study showed that the frequency and intensity of bowel sounds were equal throughout all four quadrants[4]. The diagnosis of each of the patients was determined by clinical follow-up, clinical evolution, and by radiological and operative findings. The recorded sound tracks were analysed by the main investigator using the supplied software. The investigators were not blinded to the results of other tests such as radiological imaging or intraoperative findings.

When analyzing the sound tracks, the tracks were played back in the "diaphragm" mode. The overall quality of recording was determined by the level of ambient background noise that was actually present at the time of recording, and the presence of a constant "hissing" machinery noise (not present in the environment during recording), which could sometimes occur during playback of some of the tracks. This "ghost sound" phenomenon was noticeably more common when the entire track lacked actual bowel sounds and it was thought that the recording system compensates for the lack of sound by automatic over-amplification of the baseline machine noise. The waveform view displays the raw input signal as a continuous waveform line, where each wave is a pulse of sound. The maximum arbitrary amplitude (range 0% to 100%) of the bowel sounds was identified from each track (Figure 1).

Classification of bowel sounds

The sounds were described as "isolated" when they occurred in isolation and did not last for more than 0.5 s in duration and were more than 0.2 s apart. The spike of the isolated sound is shown as a single vertical line over a very short duration (Figure 1). The sounds were described as "clustered" when they occurred continuously for more than 0.5 s and recognised audibly as a run of individual "popping" effects (Figure 1), and "prolonged" when they occurred continuously for longer than 4 s[5]. The interval between sounds was determined by the period between the end of one sound and the start of the next sound. For the purpose of computation, when there were more than two distinct bowel sounds on a single track the shortest interval between two distinct bowel sounds was taken as the interval between sounds for that track. The spectrum view displays the frequency

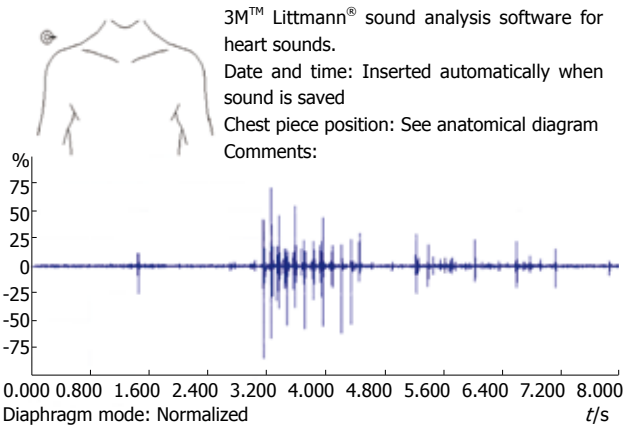

Figure 1 Example of bowel sound recording displayed in the "waveform" view. The y-axis indicates the relative sound amplitude and the x-axis indicates recording time elapsed in seconds. This recording was taken from a patient with a small bowel obstruction. Note the isolated sound on the left side followed by two clusters of sound in the middle and on right side of the strip.

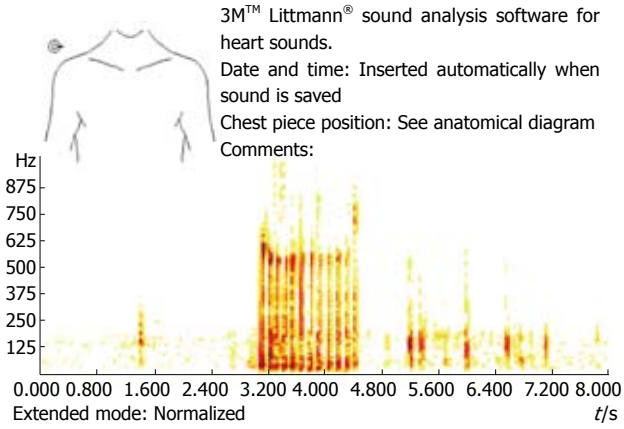

Figure 2 Example of bowel sound recording displayed in the "spectrum" view. This chart shows the sound frequency spectrum and the relative intensity of each sound frequency (darker colour indicates higher sound intensity at the frequency indicated along the y-axis) for the same recording used for Figure 1.

distribution as vertical bars, each bar represents a group of frequencies, and the more prominent frequencies have darker colour tones than the less prominent frequencies (Figure 2). The highest dominant frequency from each track was determined by first identifying the most dominant sound frequency across the spectrum at each point of time when bowel sounds were present. The highest dominant frequency over the entire duration of the track was then identified and used for analysis. The peak frequency was determined by the highest value dominant frequency among the six tracks for each individual patient.

X-rays and computed tomography (CT) imaging of the abdomen and pelvis were reviewed for evidence of intestinal distension. The calibre of the small bowel and large bowel at the widest points were determined using the measuring tool in the Amalga IMS 5.1 radiology image viewing system installed on our institution's computers. The point of transition was identified if bowel obstruction was present on CT images. Any operative intervention performed for the patients was also documented. The cause of obstruction, as identified from radiological imaging and/or during surgery, was recorded.

The following definitions were used to compare bowel sounds between patients with acute bowel obstruction *vs* subacute obstruction *vs* no obstruction[6-8]:

Acute bowel obstruction: Dilated bowel calibre (> 3 cm for small bowel, > 6 cm for large bowel) with symptoms of vomiting (for small bowel pathology) or no flatus/bowel motion for > 24 h (for large bowel pathology) and with CT/operative evidence of complete or high grade mechanical obstruction with a discrete transition point, collapsed distal segment, and little or no colonic gas present distal to the obstruction.

Subacute obstruction: Dilated bowel calibre (> 3 cm for small, > 6 cm for large bowel), with or without symptoms as per acute obstruction, but no CT/operative evidence of complete or high-grade mechanical obstruction (a poorly defined transition zone, incomplete collapse of distal bowel segment, and moderate colonic gas distal to the obstruction).

Acute (complete) or subacute (partial) mechanical obstruction of the bowel may be caused by herniation, adhesions, volvulus, intussusception, foreign body, phytobezoar, strictures, neoplasia, or wall oedema due to inflammatory conditions. Partial obstruction allows some liquid contents and gas to pass through the point of obstruction, whereas complete obstruction impedes passage of all bowel contents.

No obstruction: No clinical/CT/operative/endoscopic evidence of mechanical obstruction and other diagnosis was observed during the clinical course of the patient. Diagnoses such as constipation colic, faecal impaction, paralytic ileus, chronic megacolon, and pseudo-obstruction will be classified under this category.

Statistical analysis

Statistical analysis was performed using SPSS Version 12.0.1 (SPSS Inc. Chicago, Illinois, United States). Continuous non-parametric data were analysed using Mann-Whitney *U* test. A χ^2 test was used to correlate the type of bowel sounds with the presence of obstruction. Pearson correlation was used to correlate bowel calibre with sound duration, sound-to-sound interval, dominant frequency, and peak frequency. Continuous data was presented as median values with inter-quartile ranges.

RESULTS

Seventy-one patients were recruited and had their bowel sounds recorded during the period July 2009 to January 2011. These patients had been admitted to our institution with abdominal symptoms, physical signs, or radiological

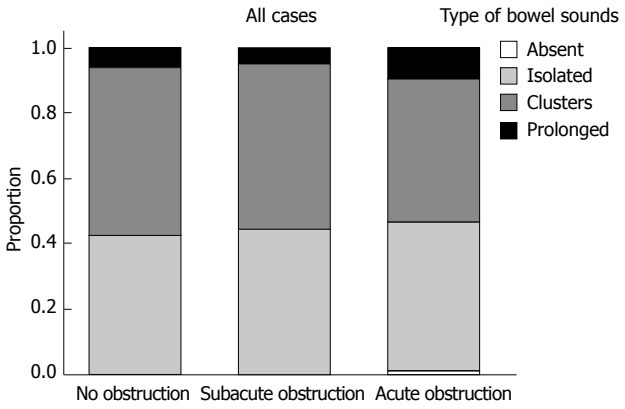

Figure 3 Distribution of different types of bowel sounds recorded for patients with no obstruction, subacute obstruction, and acute obstruction.

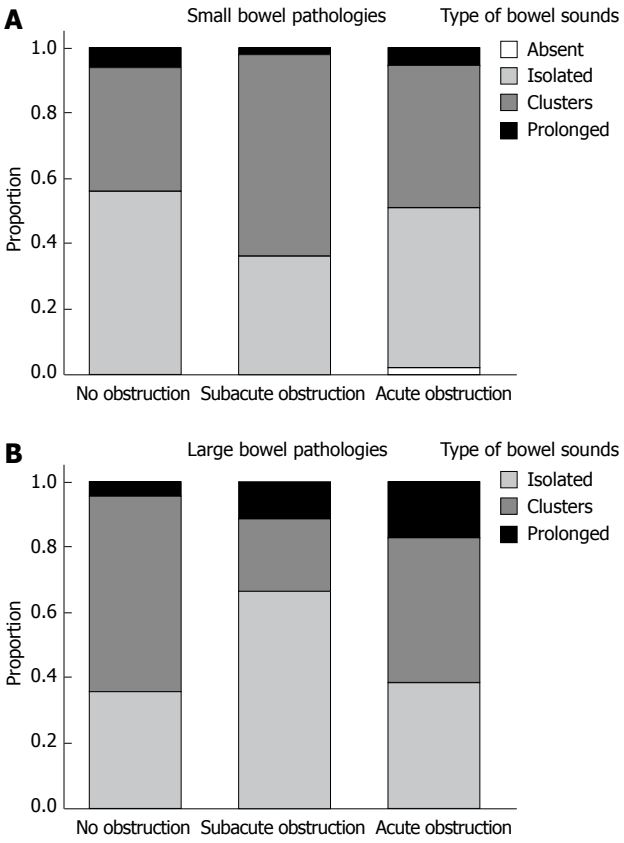

Figure 4 Distribution of different types of bowel sounds recorded for patients with small bowel pathologies (A) and large bowel pathologies (B).

findings suggestive of possible bowel obstruction.

There were 43 male and 28 female patients with age range from 22 to 93 years (mean 68 ± 16 years). Sixty-nine percent of patients presented with abdominal pain and 70.4% had a history of vomiting. Abdominal distension was present in 90.1% of the patients and abdominal tenderness was present in 33.8%. All patients had radiological imaging of the abdomen and pelvis. Plain film radiography was performed in all patients and CT was performed in 85.9% of patients.

Acute bowel obstruction was diagnosed in 40 patients (56.3%). Of these, 27 occurred in the small bowel and 13 occurred in the large bowel. Eleven patients (15.5%) had subacute bowel obstruction, of which eight occurred in the small bowel and three occurred in the large bowel. Twenty patients (28.2%) had no bowel obstruction. The diagnoses made for the non-obstructive conditions were ileus, pseudo-obstruction, gastritis, gastroenteritis, ileitis, colitis, constipation, cholecystitis, and bowel ischemia. Surgical intervention was performed in 25 patients (35.2%) during the same admission for the acute abdominal conditions.

Four hundred and twenty-six recordings were made from the 71 patients, of which 6 were of poor quality and were not used for analysis.

All types of bowel sounds were heard in all three groups of patients. Overall, isolated and clustered bowel sounds were heard most frequently in approximately equal proportions in all three groups of patients and prolonged sounds comprised 9.3% in the group with acute bowel obstruction (Figure 3).

The cases were further analysed separately in subgroups of patients with small bowel pathologies and patients with large bowel pathologies. In the subgroup of patients with small bowel pathologies, the incidence of prolonged sounds were 6%, 2% and 6% with no obstruction, subacute obstruction and acute obstruction, respectively, and were not significantly different (P = 0.208) (Figure 4A). In the subgroup of patients with large bowel pathologies, the incidence of prolonged sounds increased significantly from 4% with no obstruction to 11% with subacute obstruction, and 17% with acute obstruction (P = 0.025) (Figure 4B).

Among the groups of patients with no bowel obstruction, subacute obstruction, and acute obstruction, the distributions of dominant frequencies were similar, with the highest number of recordings with a dominant frequency in the 100 to 300 Hz range (Figure 5A). None of the recordings showed a dominant frequency of over 1000 Hz.

There was no significant difference between the sound characteristics in terms of sound duration (Figure 5B), sound-to-sound interval (Figure 5C), dominant frequency (Figure 5D), and peak frequency (Figure 5E) when comparisons were made among the groups of patients with acute bowel obstruction, subacute bowel obstruction, and no bowel obstruction. The results are summarised in Table 1.

In the patient subset with acute bowel obstruction (n = 40), 27 patients had a small bowel obstruction and 13 had a large bowel obstruction. The sound characteristics in acute small and large bowel obstruction were compared (Table 2). The sound duration was significantly longer (P = 0.021) and the dominant frequency significantly higher (P = 0.003) in the large bowel obstruction group. The median peak frequency was about 100 Hz higher in the large bowel obstruction group, but the difference did not reach statistical significance (P = 0.060).

Comparison was made between patients with acute large bowel obstruction and the three patients with

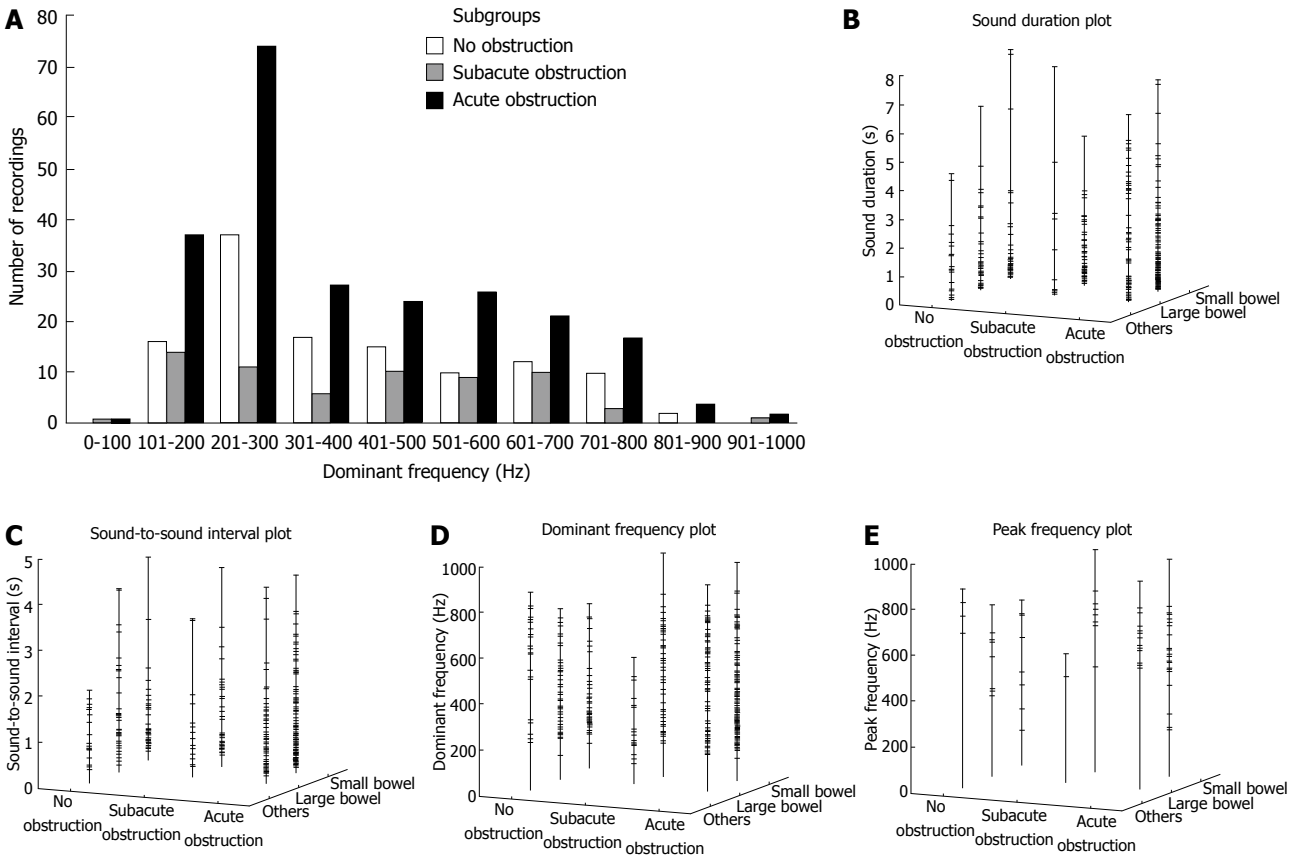

Figure 5 Distribution of the dominant frequencies of bowel sounds (A), sound duration (B), sound-to-sound interval (C), dominant frequency (D), and peak frequency (E) in patients with no obstruction, subacute obstruction, and acute obstruction, *etc*.

Table 1 Sound characteristics of acute bowel obstruction *vs* subacute bowel obstruction *vs* no obstruction

	No obstruction (n = 20)	Subacute obstruction (n = 11)	Acute obstruction (n = 40)	P value
Sound duration (s)	0.64 (0.20-1.57)	0.63 (0.23-1.67)	0.69 (0.19-2.10)	> 0.05
Sound-to-sound interval (s)	0.72 (0.46-1.27)	0.70 (0.47-1.67)	0.75 (0.41-1.41)	> 0.05
Dominant frequency (Hz)	325 (225-530)	405 (218-565)	315 (225-545)	> 0.05
Peak frequency (Hz)	595 (378-713)	655 (465-735)	585 (530-706)	> 0.05

Data are presented as the median (inter-quartile range).

Table 2 Sound characteristics of acute small bowel obstruction *vs* acute large bowel obstruction

	Small bowel obstruction (n = 27)	Large bowel obstruction (n = 13)	P value
Sound duration (s)	0.55 (0.17-1.67)	0.87 (0.27-3.60)	0.021
Sound-to-sound interval (s)	0.72 (0.41-1.40)	0.78 (0.44-1.42)	0.621
Dominant frequency (Hz)	288 (220-479)	440 (250-643)	0.003
Peak frequency (Hz)	560 (480-695)	660 (578-740)	0.060

Data are presented as the median (inter-quartile range).

pseudo-obstruction of the large bowel (Table 3). There was no significant difference in the sound characteristics between the two groups. Comparison between patients with a small bowel obstruction and a pseudo-obstruction (Table 4) showed significantly longer sound duration, longer sound-to-sound interval, and higher dominant frequency in patients with a pseudo-obstruction.

Analysis was performed on the 35 patients with a small bowel obstruction (27 acute obstruction and eight subacute obstruction included). Twenty-six patients (74%) with a small bowel obstruction were caused by (or presumed to be caused by) adhesions when there was a previous history of abdominal operation(s), CT (performed on 30 patients) did not find any mass lesion causing the obstruction, or the diagnosis confirmed during surgical exploration. Other causes of obstruction were small bowel volvulus (n = 3), bowel non-rotation (n = 1), femoral hernia (n = 1), parastomal hernia (n = 1), omental mass invasion (n = 1), and unknown (n = 2). The bowel sounds characteristics were compared between those who were treated conservatively (n = 25) *vs* those who were operated on (n = 10) (Table 5). The dominant frequency and peak frequency between non-operated and operated groups were not significantly different. The sound duration in the operated group was

Table 3 Sound characteristics of acute large bowel obstruction *vs* large bowel pseudo-obstruction

	Large bowel obstruction (n = 13)	Large bowel pseudo-obstruction (n = 3)	P value
Sound duration (s)	0.87 (0.27-3.60)	1.24 (0.53-2.64)	0.686
Sound-to-sound interval (s)	0.78 (0.44-1.42)	1.08 (0.68-2.23)	0.061
Dominant frequency (Hz)	440 (250-643)	488 (389-679)	0.174
Peak frequency (Hz)	660 (578-740)	630 (525-750)	0.638

Data are presented as the median (inter-quartile range).

Table 4 Sound characteristics of acute small bowel obstruction *vs* large bowel pseudo-obstruction

	Small bowel obstruction (n = 27)	Large bowel pseudo-obstruction (n = 3)	P value
Sound duration (s)	0.55 (0.17-1.67)	1.24 (0.53-2.64)	0.048
Sound-to-sound interval (s)	0.72 (0.41-1.40)	1.08 (0.68-2.23)	0.041
Dominant frequency (Hz)	288 (220-479)	488 (389-679)	0.001
Peak frequency (Hz)	560 (480-695)	630 (525-750)	0.446

Data are presented as the median (inter-quartile range).

longer than the non-operated group, but the difference did not reach statistical significance. The sound-to-sound interval was significantly longer in the operated group (P < 0.001).

Analysis of bowel sounds was made in relation to bowel dilatation in acutely obstructed bowel. There was no correlation between the small bowel calibre (4.8 ± 1.6 cm) and the sound duration (r = -0.046, P = 0.567), sound-to sound interval (r = -0.020, P = 0.817), dominant frequency (r = 0.025, P = 0.753), and peak frequency (r = -0.208, P = 0.298) for acute small bowel obstruction. Similarly, for acute large bowel obstruction, there was no correlation between the large bowel calibre (8.2 ± 1.7 cm) and sound duration (r = -0.103, P = 0.372), sound-to-sound interval (r = -0.077, P = 0.535), dominant frequency (r = -0.022, P = 0.847), and peak frequency (r = -0.028, P = 0.927).

DISCUSSION

Bowel sounds are generated by contractions of the alimentary tract, and mixing of gaseous and liquid contents[9]. The quality of bowel sounds varies according to the state of bowel activity[10,11]. Bowel sounds are complex, and each sound comprises a mixture of tones and is often a sequence of closely connected sounds. Common descriptions of bowel sounds include gurgling or rattling or rustling noise heard in a normal person, rumbling explosions heard with gastroenteritis, succussion splash heard in gastric outlet obstruction, diminished, i.e., infrequent, and soft sounds, and prolonged tinkling or high-pitched metallic sounds that may be heard in bowel obstruction. Very diminished or absent bowel sounds may be caused by bowel obstruction, intestinal ischemia, paralytic ileus, and peritonitis.

Table 5 Sound characteristics of non-operated and operated patients with small bowel obstruction

	Small bowel obstruction, non operated (n = 25)	Small bowel obstruction, operated (n = 10)	P value
Sound duration (s)	0.55 (0.21-1.45)	1.00 (0.25-2.26)	0.100
Sound-to-sound interval (s)	0.63 (0.39-1.13)	1.29 (0.61-1.82)	< 0.001
Dominant frequency (Hz)	360 (225-560)	265 (210-455)	0.084
Peak frequency (Hz)	625 (535-713)	525 (400-738)	0.432

Data are presented as the median (inter-quartile range).

The published literature on auscultation of bowel sounds has been rather scarce over the past century. Since Cannon described the rhythmic sounds produced by the stomach and intestines more than 100 years ago, very few papers have been published on this subject[12].

Advances in technology have allowed various systems to be developed for the objective analysis of bowel sounds. Spectral analysis of bowel sounds was first described by Horn *et al*[13] in 1966. The spectrogram that their apparatus produced was a complex record, which was difficult to understand, and the frequency range of their apparatus was too small. Work by Watson *et al*[14] in 1967 found that bowel sounds have a frequency range of at least 150 Hz to 5000 Hz, with peaks detected at frequencies of up to 2000 Hz. These very high frequencies were not seen in our study, where the peak dominant frequency of bowel sounds recorded never reached above 1000 Hz in any of our recordings.

Yoshino *et al*[15] in 1990 attempted a computer analysis of bowel sounds in intestinal obstruction. The number of subjects studied was small (n = 21). The peak frequency of patients with intestinal obstruction in the subgroup of patients that required surgery (612 ± 86 Hz, n = 5) was significantly higher compared with the subgroup that did not require surgery (273 ± 64 Hz, n = 3). The authors concluded that computer analysis of bowel sounds of mechanical obstruction could provide a very objective assessment of severity, and could help determine the treatment regimen (conservative or operative) of each patient. However, our data on patients with small bowel obstruction showed that the subgroup that subsequently underwent surgery (n = 10) did not have any significant difference in the dominant or peak frequencies when compared with the subgroup that did not undergo surgery (n = 25). Instead, there was a significantly longer sound-to-sound interval in the subgroup that underwent surgery compared with the subgroup that was treated conservatively. The explanation for this is difficult, but it may be that in situations

where the small bowel obstruction does not resolve and subsequently requires surgery, the affected bowel is more prone to ischemia and fatigue, becoming more distended and resulting in less frequent peristaltic activities and recordable sounds[10,16].

The study by Sugrue *et al*[17] in 1994 compared characteristics of bowel sounds in controls (n = 63) and patients with acute appendicitis (n = 25), acute cholecystitis (n = 15), and bowel obstruction (n = 21). This study compared sound number, duration, interval, and amplitude but not frequency of bowel sounds. It was found that in bowel obstruction, the sound duration, interval, and amplitude all significantly increased when compared to control subjects. However, in our current study, there was no significant difference in the sound duration and sound-to-sound interval between obstructed and non-obstructed bowel.

The current study is, so far, the largest series of patients with intestinal obstruction investigated for bowel sounds (n = 51). This study has shown objectively that bowel sound characteristics, in general, are not significantly different between patients with acute, subacute, or no intestinal obstruction using the commonly compared parameters, including sound duration, sound-to-sound interval, and dominant and peak frequencies. However, the obstructed large bowel has significantly longer sound duration (median 0.87 s *vs* 0.56 s) and higher dominant frequency (median 440 Hz *vs* 288 Hz) when compared to obstructed small bowel. The possible explanations for these differences are that movements within the colon usually involve less frequent peristalsis but larger volume shift with each peristalsis and, therefore, in the presence of an obstruction, a longer time elapses when fluid and gas are forced to pass through a tight stenosis. The higher sound frequency in large bowel obstruction can be explained by the fact that the majority of ileocecal valves are competent[18,19]. In the event of large bowel obstruction, the pressure rises within the "closed loop" segment of the colon proximal to the point of obstruction. Progressive distension of the colon increases the tension on the colonic wall, as well as thinning of the wall; hence, vibration is produced at a higher frequency. In small bowel obstruction, the pressure within the obstructed segment of bowel is usually limited by reflux of the small bowel contents back into the stomach (except in the presence of certain uncommon situations causing a closed loop obstruction), hence the common presentation of vomiting in small bowel obstruction.

In the small group of patients with large bowel pseudo-obstruction, the sound characteristics were similar to those with large bowel obstruction. This is not surprising, because in both conditions the large bowel can be grossly distended. The point of "obstruction" in patients with pseudo-obstruction is often caused by a high resting anal sphincter tone, which impedes the evacuation of flatus and faeces. This can be identified by digital rectal examination and the findings of a tight anus, which precludes easy entry of the examining finger. Upon entry of the finger, the rectum can be felt to be capacious and mainly gas and fluid filled.

The intrinsic difficulty with the study of bowel sounds is that it is never possible to hear or record exactly the same pattern of bowel sounds with exactly same amplitude, frequency, duration and interval repeatedly and consistently as compared to the consistent sounds produced from the heart, which has a set rhythm and sound characteristics over time. The bowel sounds heard from the same patient will also differ at different times when the patient is re-examined. Therefore intrasubject reproducibility of repeat recording was not evaluated in this study. The wide range of physiological variations means that the clinical significance of bowel sounds is limited. There is no clear evidence that high-pitched bowel sounds have clinical pertinence[20]. The recording of bowel sound yields only the sum of the motility of all areas of the alimentary track, so no statements are possible about the activity of any particular segment by comparison of sounds at different recording sites[21].

In this study, the recording of six 8-second tracts of bowel sounds performed over a few minutes may not be representative of the overall pattern over a longer period. The technical ability to perform continuous recording over a longer period is more likely to provide better representation of the whole picture, as well as improving intrasubject reproducibility. Recent biomedical engineering experimental studies have utilised bowel sounds to assess bowel motility and their techniques have shown good potential for monitoring and estimation of bowel motility[22-25]. Incorporation of these technologies into a portable electronic stethoscope might improve the recording, analysis, and interpretation of clinical data.

COMMENTS

Background

Auscultation of bowel sounds is a traditional technique for evaluating patients with abdominal symptoms. It is simple, but is generally empirical and too subjective. There is a distinct lack of clinical research to support the value of auscultation for bowel sounds.

Research frontiers

Recording of bowel sounds with objective evaluation has become possible with commercially available electronic stethoscopes. In this study, the authors aimed to determine the value of objective assessment of bowel sounds provided by an electronic stethoscope in supporting a clinical diagnosis of intestinal obstruction. Correlation with radiological and operative findings, and clinical outcome was made to identify characteristic bowel sounds that will improve the diagnostic accuracy for bowel obstruction.

Innovations and breakthroughs

This is the largest study examining bowel sounds for intestinal obstruction using a commercially available electronic stethoscope. Bowel sounds recorded for different conditions, including acute and subacute obstruction of the small bowel and large bowel, pseudo-obstruction of large bowel, and patients with no bowel obstruction were compared using measurable parameters, such as sound duration, sound-to-sound interval, dominant frequency, and peak frequency. The most important breakthroughs from this study are that large bowel obstruction has a significantly longer sound duration and higher dominant frequency than small bowel obstruction. Furthermore, bowel sounds in pseudo-obstruction of the large bowel mimic acute large bowel obstruction; therefore, auscultation is of less value in differentiating between these two conditions. Small bowel obstruction that resolved with conservative management had shorter sound-to-

sound interval than those that eventually needed surgery.

Applications

By understanding the differences in bowel sound qualities among various acute obstructive conditions of the bowel, practitioners can use their past experience and clinical judgement when auscultating and interpreting bowel sounds in an acute abdomen.

Terminology

Bowel sound duration, sound-to-sound interval, dominant frequency, and peak frequency are the main parameters used to analyse bowel sounds. The reasons for the observed differences among conditions, including acute and subacute obstruction, small and large bowel obstruction, were discussed.

Peer review

The authors assessed the value of objective analysis of bowel sounds in a unique group of patients who presented with a common, yet challenging, abdominal condition. The data presented in this study showed that auscultation of bowel sounds, seemingly so simple to perform, becomes very complex when subjected to a scientific study. Bowel sounds on their own may not be specific enough to provide a diagnosis of bowel obstruction. The combination of clinical and radiological assessments remains the standard for diagnosing bowel obstruction.

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BRIEF ARTICLE

Stopping or reducing dietary fiber intake reduces constipation and its associated symptoms

Kok-Sun Ho, Charmaine You Mei Tan, Muhd Ashik Mohd Daud, Francis Seow-Choen

Kok-Sun Ho, Charmaine You Mei Tan, Muhd Ashik Mohd Daud, Francis Seow-Choen, Seow-Choen Colorectal Surgery PLC, Singapore 238859, Singapore
Author contributions: Seow-Choen F designed the research; Tan CYM and Mohd Daud MA were responsible for data collection and analysis; Ho KS and Seow-Choen F wrote the paper.
Correspondence to: Francis Seow-Choen, MBBS, FRCSEd, FAMS, Seow-Choen Colorectal Surgery PLC, 290 Orchard Road, The Paragon No. 06-06, Singapore 238859, Singapore. seowchoen@colorectalcentre.com
Telephone: +65-67386887 Fax: +65-67383448
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Abstract

AIM: To investigate the effect of reducing dietary fiber on patients with idiopathic constipation.

METHODS: Sixty-three cases of idiopathic constipation presenting between May 2008 and May 2010 were enrolled into the study after colonoscopy excluded an organic cause of the constipation. Patients with previous colon surgery or a medical cause of their constipation were excluded. All patients were given an explanation on the role of fiber in the gastrointestinal tract. They were then asked to go on a no fiber diet for 2 wk. Thereafter, they were asked to reduce the amount of dietary fiber intake to a level that they found acceptable. Dietary fiber intake, symptoms of constipation, difficulty in evacuation of stools, anal bleeding, abdominal bloating or abdominal pain were recorded at 1 and 6 mo.

RESULTS: The median age of the patients (16 male, 47 female) was 47 years (range, 20-80 years). At 6 mo, 41 patients remained on a no fiber diet, 16 on a reduced fiber diet, and 6 resumed their high fiber diet for religious or personal reasons. Patients who stopped or reduced dietary fiber had significant improvement in their symptoms while those who continued on a high fiber diet had no change. Of those who stopped fiber completely, the bowel frequency increased from one motion in 3.75 d (± 1.59 d) to one motion in 1.0 d (± 0.0 d) ($P < 0.001$); those with reduced fiber intake had increased bowel frequency from a mean of one motion per 4.19 d (± 2.09 d) to one motion per 1.9 d (± 1.21 d) on a reduced fiber diet ($P < 0.001$); those who remained on a high fiber diet continued to have a mean of one motion per 6.83 d (± 1.03 d) before and after consultation. For no fiber, reduced fiber and high fiber groups, respectively, symptoms of bloating were present in 0%, 31.3% and 100% ($P < 0.001$) and straining to pass stools occurred in 0%, 43.8% and 100% ($P < 0.001$).

CONCLUSION: Idiopathic constipation and its associated symptoms can be effectively reduced by stopping or even lowering the intake of dietary fiber.

Key words: Dietary fiber; Constipation; Chronic idiopathic constipation; Abdominal bloating

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INTRODUCTION

Lack of fiber in the diet was first postulated in 1971

as the cause of diseases such as diverticulosis, hemorrhoids and colorectal cancer[1]. Since then, partly due to widespread media publicity, it is now widely accepted that dietary fiber is a necessary component of a healthy diet and is required for normal bowel movement[2-5]. It is popularly used in the management of constipation by the public and by many doctors. Insoluble fiber is known to increase stool weight and decrease colonic transit time[6,7]. Fiber is said to aid in water retention in the colon and results in stools that are less dry and easier to evacuate. However, the reality is that stool moisture content remains at 70%-75% regardless of the amount of fiber and water consumed[7,8].

There is recent evidence that low fiber intake does not equate to constipation[9]. Patients with chronic constipation also have similar fiber intake to controls[10-13]. Patients with chronic constipation may also have worsening symptoms when dietary fiber intake is increased[14]. Another study found that lactulose was more effective in easing constipation when compared with fiber[15].

It has also been our experience that many patients with constipation are already consuming a large amount of fiber before they seek medical attention.

We therefore carried out a prospective longitudinal case study to investigate the effect of decreasing dietary fiber in patients with idiopathic constipation.

MATERIALS AND METHODS

Constipation was defined clinically in patients who presented either with symptoms of straining to expel bulky large stools or a bowel frequency of less than one motion per 3 d over a period of at least 3 mo. Patients who presented to the clinic with symptoms of constipation, abdominal distension, pain or bloating, difficulty in evacuation with or without symptoms of rectal bleeding were considered for the study. For the purpose of this study, we did not distinguish between slow colonic transit type or obstructed defecation type of constipation nor did we attempt to classify the patients according to irritable bowel syndrome subtypes. All the patients underwent colonoscopy to exclude colonic lesions. Patients who had colorectal cancer, previous colonic surgery, melanosis coli or thyroid disorders were excluded. Patients with anal conditions such as severe prolapsed hemorrhoids, chronic anal fissure or any other condition that required surgery were also excluded.

Sixty-three consecutive patients after normal colonoscopy were enrolled into the study from May 2008 to May 2010. Each patient was to act as their own control. The physiology of the gastrointestinal tract and the bulking effects of dietary fiber were explained to the patients[16,17]. Patients were then instructed to completely stop their intake of dietary fiber, including vegetables, cereals, fruits, wholemeal bread and brown rice for 2 wk. Those who were vegetarians were asked to eat white rice instead of unpolished rice, white bread instead of whole meal bread, and to take processed bean products for protein. They were to continue their normal quantities of carbohydrates and proteins. Sieved fruit juices and clear vegetable soups were allowed. Patients were instructed not to take any laxatives during these 2 wk. After 2 wk, patients were asked to continue on with as little fiber in their diet as they were comfortable with for the long term. Patients were followed up at 1 mo and 6 mo intervals and final results were analyzed at 6 mo.

Data collected included age, sex, general dietary fiber intake, symptoms of constipation, difficulty in evacuation of stools, anal bleeding, abdominal bloating or abdominal pain. Constipation was recorded as the interval in days between bowel movements. Difficulty in evacuation was a subjective measure and patients were asked to choose from one of 3 degrees (no straining, occasional or moderate straining and severe straining or straining most of the time).

Statistical analysis

All data was entered into a secured database, and accessed only by the authors. The paired samples *t* test was performed using SPSS for Windows (SPSS Inc., Chicago, United States), version 17.0 on an IBM personal computer. Results are expressed as mean ± SD.

RESULTS

There were 16 males (25.6%) and 47 (74.4%) females, median age 47 years (range, 20-80 years) included in the study. At the commencement of the study, all patients were already on a high fiber diet or taking fiber supplements. After 2 wk of a no fiber diet, patients were asked to continue on as little fiber in the diet as they were able to follow if this were to give them relief from their symptoms.

At 6 mo, 41 patients continued on a no fiber diet and 16 were on a reduced fiber diet. The remaining 6 patients continued on a high fiber diet for various reasons including being vegetarians or inability to stop consuming dietary fiber for religious or personal reasons.

The median age of patients who stayed on a no fiber diet was 46 years (range, 21-80 years), on a reduced fiber diet was 45 years (range, 20-65 years) and on a high fiber diet was 59 years (range, 28-75 years). There was no statistical significant difference in age between the 3 groups. There was also no statistical difference in sex between the 3 groups (Table 1).

At 6 mo follow-up, the interval between bowel movements decreased with the reduction in fiber intake ($P < 0.001$). Forty one patients who completely stopped fiber intake had their bowel frequency increased from one motion in 3.75 d (± 1.59 d) to one motion in 1.0 d (± 0.00 d) ($P < 0.001$). Of 16 patients who reduced their dietary fiber intake, 12 patients had daily bowel movement, 3 had one bowel movement every 2 to 3 d and one had a bowel movement every 4 to 6 d, giving one motion per 1.9 d (± 1.21 d) on a reduced fiber diet compared with 1 motion per 4.19 d (± 2.09 d) on a high fiber diet ($P < 0.001$). There was no change in the frequency of bowel movement for patients who continued with high dietary fiber intake, with one motion per 6.83 d (± 1.03 d) before and after consultation ($P = 1.00$).

Table 1 Age and sex of all patients, segregated by post-consultation dietary fiber intake

Variable	General (n = 63)	No fiber (n = 41)	P value	Reduced fiber (n = 16)	P value	High fiber (n = 6)	P value
Age (yr), mean (range)	47 (20-80)	46 (21-80)	0.864	45 (20-65)	0.459	59 (28-75)	0.052
Sex[1] n (%)	16 (25.4)	15 (36.6)	0.258	1 (6.25)	0.034	0 (0)	< 0.01

[1]Numbers of male in each group. Corresponding P values for the age and sex of each category to the overall mean are provided.

Table 2 Symptoms at presentation and at 6 mo following change in dietary fiber intake

Symptom	Symptoms at presentation (n = 63)	High dietary fiber (n = 6)	P value	Reduced dietary fiber (n = 16)	P value	No dietary fiber (n = 41)	P value
Anal bleeding	31	4	1	4	0.216	0	< 0.001
Constipation	63	6	1	12	0.041	0	< 0.001
Bloatedness	33	6	1	5	0.041	0	< 0.001
Strain in bowel opening	63	6	1	9	0.004	0	< 0.001
Abdominal pain	13	3	1	2	0.164	0	0.012

There was also a difference between the groups in the proportion of patients with associated symptoms. For symptoms of bloating, all of those on a high fiber diet continued to be symptomatic, while only 31.3% in the reduced fiber group and none of the no fiber group had symptoms (0%, $P < 0.001$) (Table 2).

With regards to straining, all those on a no fiber no longer had to strain to pass stools. Of those who reduced dietary fiber, 7 of 16 showed improvement while the symptoms remain unchanged in those who remained on a high fiber diet ($P < 0.001$ between groups).

Symptoms of abdominal pain only improved in patients who stopped fiber completely while those who continued on a high fiber diet or reduced fiber diet did not show any improvement (Table 2). In addition, those on a no dietary fiber diet no longer had symptoms of anal bleeding.

DISCUSSION

This study has confirmed that the previous strongly-held belief that the application of dietary fiber to help constipation is but a myth. Our study shows a very strong correlation between improving constipation and its associated symptoms after stopping dietary fiber intake. However whilst there was no significant difference between the mean age of the 3 groups with different post-consultation dietary fiber intake, older patients seemed less likely to stop dietary fiber, although this did not reach significance. We did not survey the actual reasons for resuming dietary fiber. The clinical impression during consultation however was that some of these patients were vegetarians, some felt uneasy not eating any fiber, whilst others could not completely discontinue fiber due to constant media and peer pressure to increase dietary fiber.

Constipation is often mistaken by the layman as the state of not passing stool, with the subsequent false notion that making more feces will allow easier defecation. In truth, constipation refers to the difficulty in evacuating a rectum packed with feces, and easier defecation cannot possibly be affected by increasing dietary fiber which increases bulky feces. In this paper, we looked at constipation both as the number of days before each motion as well as the ease of defecation.

It is well known that increasing dietary fiber increases fecal bulk and volume. Therefore in patients where there is already difficulty in expelling large fecal boluses through the anal sphincter, it is illogical to actually expect that bigger or more feces will ameliorate this problem. More and bulkier fecal matter can only aggravate the difficulty by making the stools even bigger and bulkier. Several reviews and a meta-analysis had already shown that dietary fiber does not improve constipation in patients with irritable bowel diseases[18-21].

The role of dietary fiber in constipation is analogous to cars in traffic congestion. The only way to alleviate slow traffic would be to decrease the number of cars and to evacuate the remaining cars quickly. Should we add more cars, the congestion would only be worsened. Similarly, in patients with idiopathic constipation and a colon packed with feces, reduction in dietary fiber would reduce fecal bulk and volume and make evacuation of the smaller and thinner feces easier. Adding dietary fiber would only add to the bulk and volume and thus make evacuation even more difficult.

Whilst it is often stated in physiology textbooks that bulking agents improve peristalsis, there is no proof of this in practice nor experimentally. Regardless of the food ingested, small intestinal and right mid colonic contents are fluid and all ingestible dietary fiber is suspended therein. Dietary fiber, therefore, cannot act as solid boluses for the initiation of peristalsis. In fact, dietary fiber had been shown to retard peristalsis and hold up gaseous expulsion in human experiments[22].

Dietary fiber is also associated with increased bloatedness and abdominal discomfort[22]. Insoluble fiber was reported to worsen the clinical outcome of abdominal pain and constipation[18-20]. In our recent study, patients who followed a diet with no or less dietary fiber intake showed a significant improvement, not just in their constipation,

but also in their bloatedness. Patients who completely stopped consuming dietary fiber no longer suffered from abdominal bloatedness and pain. These symptoms are caused by the fermentation of dietary fiber by colonic bacteria, which produces hydrogen, carbon dioxide and methane[23]. Gases that are trapped by peristaltic colon exert pressure on the walls, causing the abdominal pain experienced by patients. This was previously observed in a prior study on younger patients, when dietary fiber had been shown not to be effective in the management of children with recurrent abdominal pain or bloating[21].

Stools only become well-formed in the sigmoid colon and rectum and by this time, especially in constipated subjects, more stools result in more evacuation problems. It is not logical to increase both the volume and size of stool in patients with idiopathic constipation and indeed for anybody with difficulty in passing stools, e.g., due to anismus or anal spasm from anal stricture, fissure or pelvic outlet disorders. We have shown that decreasing the bulk and volume of feces immediately enables the easier evacuation of smaller and thinner stools through the anal sphincter mechanism. This eliminates the need to strain in passing stools, and prevents the tearing of the anal sphincter and bleeding due to large and bulky fecal loads. None of our patients experienced anal bleeding or straining following complete abstinence from dietary fiber.

The results of this study should lead us to reexamine popular beliefs in benefits of dietary fiber and more studies should be undertaken to confirm or repudiate these results.

In conclusion, contrary to popularly held beliefs, reducing or stopping dietary fiber intake improves constipation and its associated symptoms.

COMMENTS

Background

It is a widely accepted view that dietary fiber is essential for gut health and to promote bowel movements. However, most patients with chronic constipation seen by the authors were already taking high fiber diet with no improvement in their symptoms.

Research frontiers

The role of dietary fiber in patients with chronic constip is reevaluated.

Innovations and breakthroughs

The authors showed that reducing dietary fiber intake may actually improve symptoms of chronic constipation.

Applications

This could bring relief to millions of people suffering from chronic constipation in that reducing their dietary fiber intake may relieve their symptoms and suffering.

Peer review

This paper is a preliminary study looking at the effects of dietary fiber on the symptoms of constipation on a small cohort of cases. The discussion provided an overview of the evidence available in the literature on this topic and gave a fresh perspective on the benefits or harm of excessive dietary fiber. All in all, this is an interesting preliminary study that warrants publication and further research.

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BRIEF ARTICLE

Gastric mucin expression in *Helicobacter pylori*-related, nonsteroidal anti-inflammatory drug-related and idiopathic ulcers

Doron Boltin, Marisa Halpern, Zohar Levi, Alex Vilkin, Sara Morgenstern, Samuel B Ho, Yaron Niv

Doron Boltin, Zohar Levi, Alex Vilkin, Yaron Niv, Department of Gastroenterology, Rabin Medical Center, Tel Aviv University, Petah Tikva 49100, Israel
Marisa Halpern, Department of Pathology, Rabin Medical Center, Hasharon Hospital, Petah Tikva 49100, Israel
Sara Morgenstern, Department of Pathology, Rabin Medical Center, Beilinson Hospital, Petah Tikva 49100, Israel
Samuel B Ho, Department of Medicine, University of California, and VA San Diego Healthcare System, San Diego, CA 92161, United States
Author contributions: Boltin D acquired, analysed and interpreted the data and drafted the manuscript; Halpern M and Morgenstern S interpreted the histology; Levi Z performed the statistical analysis; Vilkin A and Ho SB critically reviewed the manuscript and provided important intellectual content; Niv Y supervised the study, assisted in the drafting of the manuscript, obtained funding and provided administrative, technical support.
Supported by Beilinson Hospital Gastroenterology Department Trust Fund
Correspondence to: Yaron Niv, Professor, Department of Gastroenterology, Rabin Medical Center, Tel Aviv University, 39 Jabotinski Street, Petah Tikva 49100, Israel. nivyaron80@gmail.com
Telephone: +972-3-9377237 Fax: +972-3-9210313
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Abstract

AIM: To determine the pattern of secreted mucin expression in *Helicobacter pylori* (*H. pylori*)-related, nonsteroidal anti-inflammatory drug (NSAID)-related and idiopathic gastric ulcers.

METHODS: We randomly selected 92 patients with *H. pylori*-associated (n = 30), NSAID-associated (n = 18), combined *H. pylori* and NSAID-associated gastric ulcers (n = 24), and patients with idiopathic gastric ulcers (n = 20). Immunohistochemistry for T-cell CD4/CD8, and for mucin 5AC (MUC5AC) and mucin 6 (MUC6), was performed on sections of the mucosa from the ulcer margin. Inflammation score was assessed according to the Sydney system.

RESULTS: MUC5AC was expressed on the surface epithelium (98.9%) and neck glands (98.9%) with minimal expression in the deep glands (6.5%). MUC6 was strongly expressed in the deep glands (97.8%), variable in the neck glands (19.6%) and absent in the surface epithelium (0%). The pattern of mucin expression in idiopathic ulcer margins was not different from the expression in ulcers associated with *H. pylori*, NSAIDs, or combined *H. pylori* and NSAIDs. CD4/CD8 ratio was higher in *H. pylori*-positive patients (P = 0.009). Idiopathic ulcers are associated with hospitalized patients and have higher bleeding and mortality rates.

CONCLUSION: Idiopathic ulcers have a unique clinical profile. Gastric mucin expression in idiopathic gastric ulcers is unchanged compared with *H. pylori* and/or NSAID-associated ulcers.

Key words: Idiopathic ulcer; Mucin; Mucin 5AC; Mucin 6; *Helicobacter pylori*

Peer reviewer: Waliul Khan, MBBS, PhD, Mcmaster University, 1200 Main Street West, Hamilton, Ontario L8N 3Z5, Canada

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INTRODUCTION

Helicobacter pylori (*H. pylori*) infection and non-steroidal anti-inflammatory drugs (NSAIDs) are the leading causes of peptic ulcer[1-5]. However, in up to 39% of cases neither of these risk factors is identified[6]. While it may be prudent to exclude rarer causes of gastric ulcer such as malignancy, Zollinger-Ellison syndrome or systemic mastocytosis, in many cases *H. pylori*/NSAID-negative ulcers are apparently idiopathic.

Mucins are high-molecular-weight glycoproteins, which are heavily decorated with O-linked oligo-saccharides and n-glycan chains, linked to a protein backbone. There are 21 mucin (*MUC*) genes known in the human genome. These genes encode 2 groups of mucins: secreted mucins and membrane-bound mucins. The main mucins expressed in the stomach are MUC 1 (membrane-bound) and MUC5AC and MUC6 (secreted mucins). It has been proposed that defects in gastric mucin quality or quantity play a role in the pathogenesis of *H. pylori*/NSAID-negative ulcers[7]. MUC5AC, forming the bulk of the adherent unstirred mucous layer, is secreted by surface foveolar cells, whereas MUC6 is secreted by neck and gland cells, and both are strongly expressed in normal gastric mucosa[8]. These two mucin proteins remain segregated within the mucous gel in a laminated linear arrangement[9]. NSAIDs disrupt the production of prostaglandin-E2 which mediates mucin secretion. *H. pylori* similarly decreases mucin synthesis *via* inhibition of galactosyltransferase[10], this despite mucin's inherent antibacterial properties[11].

The pattern of mucin secretion in idiopathic peptic ulcer disease has yet to be determined. The aim of the present study is to identify the clinical and endoscopic features, and gastric mucin secretion patterns, in peptic ulcer disease positive and negative for *H. pylori* infection and/or NSAID therapy.

MATERIALS AND METHODS

Patients

Approval was granted by the ethics committee of Rabin Medical Center. Non-consecutive patients who underwent routine or emergency upper endoscopy at the Department of Gastroenterology, Rabin Medical Center, Beilinson Hospital, and who were assigned an endoscopic diagnosis of gastric ulcer, between 2003 and 2009, were randomly identified using an established computerized endoscopy reporting system. Clinical parameters were recorded, including patient age and gender, major indication for upper endoscopy, concomitant diseases and the use of aspirin and NSAIDs in the previous 3 mo. Endoscopic parameters were recorded, including ulcer site, size and number. *H. pylori* status was determined *via* histological detection on biopsies taken from the ulcer margins, gastric body or antrum and/or the rapid urease test and/or ^{13}C-urea breath test performed within 3 mo. Exclusion criteria included cases where no biopsies were taken from the ulcer margin, and where biopsies revealed neoplasia. Patients with clinical or histological evidence of Zollinger-Ellison syndrome, gastrointestinal malignancy, eosinophilic gastroenteritis, systemic mastocytosis, and patients receiving bisphosphonates, potassium salts or iron, were excluded.

Tissue samples

Formalin-fixed, paraffin-embedded tissue samples from ulcer margins were obtained from the Pathology Department. Where additional biopsies of gastric body or antrum were taken at endoscopy, these too were obtained. Paraffin-embedded blocks were cut into 4 μm thick sections. Slides were deparaffinized in xylene and rehydrated using a graded ethanol series. Antigen was retrieved by boiling the slides in a microwave oven for 15 min in 0.01 mol/L citrate buffer (pH 6.0). Endogenous peroxidase was blocked with a 3% H_2O_2-methanol solution, and the slides were incubated in 10% normal goat serum for 30 min to prevent nonspecific staining. The tissue sections were then incubated overnight at 4 ℃ with primary antibody (MUC5AC or MUC6, 1:100; Santa Cruz, CA). The standard biotin-streptavidin-peroxidase method was then used, and the sections were lightly counterstained with hematoxylin. Histologically normal gastric biopsies were used as positive controls for MUC5AC and MUC6. The sections incubated with phosphate-buffered saline (0.01 mol/L, pH 7.4) instead of primary antibody were used as negative controls. Cytoplasm staining was assessed in at least 10 high-power fields by two blinded observers at 3 sites: the foveola, the mucous neck cells, and the glands. The range of cytoplasmic staining (0: 0%-5%; 1: 6%-30%; 2: 31%-60%; and 3: 61%-100%) and the intensity of staining (0: No staining; 1: Weak staining; 2: Intermediate staining; 3: Strong staining) were assessed, and averages of the grades were taken. The final staining score was defined as the product of scores for the range and intensity of cytoplasmic staining. Staining was defined as negative if the staining score was 0 or 1, intermediate for 2, 3 or 4, and high for 6 or 9[12]. All specimens were scored blindly.

Immunohistochemistry with monoclonal antibodies to T-cell CD4 and CD8 antigens was performed for 5 cases from each group using a technique previously described[13]. Tissue sections were cut 4 μm thick from routinely processed formalin-fixed and paraffin-embedded blocks. The slides were oven dried overnight at 60 ℃. The slides were then put inside the Ventana (Benchmark, United States). The Ventana was activated by loading the pre-programmed recipe file for the appropriate antibody. For CD4 we used polyclonal antibody (Spring, CA, United States), and for CD8 a monoclonal antibody, clone SP16 (DBS, CA, United States). Immunohistochemical staining was performed by the I-view DAB detection kit of Ventana. Dark brown staining was defined as positive, and no staining was defined as negative. Staining was scored as follows: 0 (no detectable staining); 1 (1%-10% positive cells); 2 (11%-50%); 3 (51%-80%); and 4 (more than 80%). In cells with positive staining, the staining was intense and uniform, so intensity was not factored

Table 1 Patient characteristics

	H. pylori+/NSAID- *n* = 30 (32.6%)	*H. pylori*-/NSAID+ *n* = 18 (19.6%)	*H. pylori*+/NSAID+ *n* = 24 (26.1%)	*H. pylori*-/NSAID- *n* = 20 (21.7%)	Total *n* = 92
Age, yr [mean (SD, range)]	58.6 (18.58, 18-88)	72.17 (10.44, 56-89)	69.46 (13.08, 24-83)	70.3 (12.64, 42-95)	66.6 (15.5, 18-95)
Gender (male) *n* (%)	10 (33)	9 (50)	13 (54.2)	12 (60)	44 (47.8)
Ethnicity *n* (%)					
Israeli (Jewish)	11 (36.7)	9 (50)	12 (50)	6 (30)	38 (41.3)
Israeli (Arab)	3 (10)	0 (0)	2 (8.3)	1 (5)	6 (6.5)
Western Europe/United States	7 (23.3)	0 (0)	1 (4.2)	2 (10)	10 (10.9)
Eastern Europe/FSU	2 (6.7)	6 (33.3)	6 (25)	6 (30)	20 (21.7)
Middle east/Africa	7 (23.3)	2 (11.1)	3 (12.5)	4 (20)	16 (17.4)
South America	0 (0)	1 (5.6)	0 (0)	1 (5)	2 (2.2)
Inpatient *n* (%)	11 (36.7)	12 (60)	10 (41.7)	16 (80)	49 (53.3)
Comorbid disease *n* (%)					
ASCVD	3 (10)	12 (66.7)	11 (45.8)	6 (30)	32 (34.8)
COPD	2 (6.7)	4 (22.2)	2 (8.3)	7 (35)	15 (16.3)
Diabetes	4 (13.3)	4 (22.2)	12 (50)	6 (30)	26 (28.3)
Current malignancy	1 (3.3)	2 (11.1)	2 (8.3)	2 (10)	7 (7.6)
Alcohol abuse	0 (0)	0 (0)	0 (0)	3 (15)	3 (3.3)
Other significant systemic disease	1 (3.3)[3]	1 (5.6)[4]	1 (4.2)[4]	5 (25)[5]	8 (8.7)
Total[1]	7 (23.3)	15 (83.3)	19 (79.2)	14 (70)	55 (59.8)
Primary indication for endoscopy *n* (%)					
Iron deficiency anemia	9 (30)	5 (27.8)	6 (25)	1 (5)	21 (22.8)
Epigastric pain/GERD	8 (26.7)	6 (33.3)	4 (16.5)	4 (20)	22 (23.9)
Upper GI bleeding	7 (23.3)	6 (33.3)	6 (25)	9 (45)	28 (30.4)
Fecal occult blood	2 (6.7)	0 (0)	1 (4.2)	1 (5)	4 (4.3)
Weight loss	2 (6.7)	1 (5.6)	5 (20.8)	0 (0)	8 (8.7)
Screening for gastric cancer	2 (6.7)	0 (0)	0 (0)	0 (0)	2 (2.2)
Esophageal varices	0 (0)	0 (0)	0 (0)	2 (10)	2 (2.2)
Vomiting	0 (0)	0 (0)	0 (0)	1 (5)	1 (1.1)
Other[2]	0 (0)	0 (0)	2 (8.3)	2 (10)	4 (4.3)
Hemoglobin, g/dL [mean (SD, range)]	11.0 (3.22, 3.4-16.7)	10.6 (2.77, 5.5-15.5)	11.0 (2.84, 5.7-14.7)	11.1 (2.70, 6.2-15.3)	10.9 (2.98, 3.4-16.7)
Died within 12 mo of endoscopy	3 (10)	2 (11.1)	2 (8.3)	5 (25)	12 (13.0)

[1]Number of patients with comorbidities as listed; [2]Fever unknown origin, gastric outlet obstruction, and surveillance following resection of gastric and esophageal carcinoma (1 case for each indication); [3]Inflammatory bowel disease; [4]Hemodialysis; [5]Inflammatory bowel disease, sepsis, cirrhosis (2 cases), tetraplegia following trauma. *H. pylori*: *Helicobacter pylori*; NSAID: Non-steroidal anti-inflammatory drugs; ASCVD: Atherosclerotic cardiovascular disease; COPD: Chronic obstructive pulmonary disease; GERD: Gastro esophageal reflux disease; GI: Gastrointestinal. +: Positive; -: Negative.

into the scoring. The ratio of CD4/CD8 intraepithelial/mucosal lymphocytes was assessed for ten low-power microscopic fields. Inflammation score was measured according to the Sydney system, and compared between the groups[14]. Sydney score for inflammation is the sum of 5 criteria: *H. pylori* status, atrophy, intestinal metaplasia, lymphocytic infiltration and polymorphonuclear cell infiltration. Every criterion has 0 to 3 score (none exists, mild, moderate, severe) thus the range is 0 to 15.

Statistical analysis

Statistical analysis was performed using statistical package for the social sciences software 19.0 (SPSS, Inc.). Patient groups were compared using the Pearson χ^2 test, *F* test and Duncan test. *P* values were considered significant when $\leqslant 0.05$.

RESULTS

Ulcer biopsies from 92 patients were included in the final set analysis, including 30 *H. pylori*-associated ulcers, 18 NSAID-associated, 24 associated with combined *H. pylori*/NSAID and 20 idiopathic, neither associated with *H. pylori* nor NSAID use. Patient characteristics are summarized in Table 1.

Forty-four patients were male, with no significant differences between the 4 groups ($P = 0.24$). Mean age was 66.6 years. Patients with *H. pylori*-associated ulcers not receiving NSAIDs were significantly younger compared to the other 3 groups ($\alpha = 0.05$, Duncan test). There was no significant difference in the patients' origin between the groups. Although significant co-morbidities were observed in all groups, 80% (16/20) of patients with *H. pylori*/NSAID-negative ulcers were inpatients at the time of their endoscopy, compared to 45.8% (33/72) in other groups ($P = 0.007$). Furthermore, idiopathic ulcers were associated with decreased survival, where 25% (5/20) died within 12 mo, compared to 9.7% (7/72) in other groups (all cause mortality, $P = 0.04$).

Patients with *H. pylori*/NSAID-negative ulcers more often presented with upper gastrointestinal bleeding: 45% (9/20) compared with 26.4% (19/72) in the other groups ($P = 0.11$). Subacute and asymptomatic presentations (iron deficiency anemia, weight loss, fecal occult blood and screening) were less common in those with *H. pylori*/NSAID-negative ulcers (2/20, 10%, compared to 34/72,

Table 2 Endoscopic findings

	H. pylori+/NSAID- *n* = 30 (32.6%)	*H. pylori*-/NSAID+ *n* = 18 (19.6%)	*H. pylori*+/NSAID+ *n* = 24 (26.1%)	*H. pylori*-/NSAID- *n* = 20 (21.7%)	Total *n* = 92
Ulcer number (per procedure) *n* (%)					
1	21 (70)	10 (55.6)	13 (54.2)	14 (70)	58 (63.0)
2	4 (13.3)	3 (16.7)	5 (20.8)	3 (15)	15 (16.3)
⩾ 3	5 (16.7)	5 (27.8)	6 (25)	3 (15)	19 (20.7)
Ulcer size (*n*)[1]					
⩽ 5 mm	18	14	27	14	73
6-10 mm	7	8	10	11	36
11-20 mm	5	3	4	4	16
> 20 mm	4	0	0	2	6
Not specified	2	2	2	0	6
Ulcer location *n* (%)					
Antrum	19 (63.3)	10 (55.6)	18 (75)	16 (80)	63 (68.5)
Body	9 (30)	6 (33.3)	6 (25)	4 (20)	25 (27.2)
Cardia	2 (6.7)	2 (11.1)	0 (0)	0	4 (4.3)
Ulcer *H. pylori* positive *n* (%)	28 (93.3)[2]	0 (0)	20 (83.3)	0 (0)	48 (52.2)
Ulcer IM *n* (%)	5 (16.7)	4 (22.2)	3 (16.7)	4 (20)	16 (17.4)

[1]In 10 instances the endoscopist reported "ulcer number" as "multiple". Therefore the total number of ulcers is unknown and percentages cannot be calculated; [2]Cases where *H. pylori* was absent from ulcer margin, but had either positive rapid urease test, positive C13-urea breath test or positive histology in biopsy not taken from ulcer margin. NSAID: Non-steroidal anti-inflammatory drugs; *H. pylori*: *Helicobacter pylori*; IM: Intestinal metaplasia. +: Positive; -: Negative.

Table 3 Staining positivity for mucin 5AC and mucin 6 *n* (%)

		Mucin 5AC			Mucin 6		
		Surface cell positivity	Neck cell positivity	Gland cell positivity	Surface cell positivity	Neck cell positivity	Gland cell positivity
H. pylori+/NSAID-	a	29 (96.7)	29 (96.7)	2 (6.7)	0 (0)	4 (13.3)	29 (96.7)
(*n* = 30)	b	29 (96.6)	27 (90)	0 (0)	0 (0)	1 (3.3)	25 (83.3)
H. pylori-/NSAID+	a	18 (100)	18 (100)	2 (11.1)	0 (0)	4 (22.2)	17 (94.4)
(*n* = 18)	b	16 (88.9)	15 (83.3)	0 (0)	0 (0)	2 (11.1)	14 (77.8)
H. pylori+/NSAID+	a	24 (100)	24 (100)	2 (8.3)	0 (0)	5 (20.8)	24 (100)
(*n* = 24)	b	24 (100)	23 (95.8)	2 (8.3)	0 (0)	2 (8.3)	22 (91.7)
H. pylori-/NSAID-	a	20 (100)	20 (100)	0 (0)	0 (0)	5 (25)	20 (100)
(*n* = 20)	b	19 (95)	16 (80)	0 (0)	0 (0)	1 (5)	19 (95)
Total	a	91 (98.9)	91 (98.9)	6 (6.5)	0 (0)	18 (19.6)	90 (97.8)
(*n* = 92)	b	88 (95.7)	81 (88.0)	2 (2.2)	0 (0)	6 (6.5)	80 (97.0)

a: Positive stain defined as a stain extent and intensity score product ⩾ 2; b: Strongly positive stain, defined as a stain extent and intensity score product equalling 6 or 9. *H. pylori*: *Helicobacter pylori*; NSAID: Non-steroidal anti-inflammatory drugs. +: Positive; -: Negative.

Table 4 Effect of *Helicobacter pylori* and non-steroidal anti-inflammatory drug status on mucin staining

	H. pylori+	*H. pylori*-	*P* value	NSAID+	NSAID-	*P* value
MUC5AC						
Surface cell positivity	53/54 (98.1)	38/38 (100)	0.39	42/42 (100)	49/50 (98)	0.36
Neck cell positivity	53/54 (98.1)	38/38 (100)	0.39	42/42 (100)	49/50 (98)	0.36
Gland cell positivity	4/54 (7.4)	2/38 (5.3)	0.68	4/42 (9.5)	2/50 (4)	0.29
MUC6						
Surface cell positivity	0/54 (0)	0/38 (0)	NA	0/42 (0)	0/50 (0)	NA
Neck cell positivity	9/54 (16.7)	9/38 (23.7)	0.40	9/42 (21.4)	9/50 (18)	0.68
Gland cell positivity	53/54 (98.1)	37/38 (97.4)	0.80	41/42 (97.6)	49/50 (98)	0.90

H. pylori: *Helicobacter pylori*; NSAID: Non-steroidal anti-inflammatory drugs; NA: Not available. +: Positive; -: Negative.

47.2%, $P = 0.003$).

No difference between groups was observed regarding ulcer number or size. *H. pylori*-negative/NSAID-positive ulcers tended to be more often located in the gastric body: 6/18 (33.3%) compared with other groups 19/74 (25.7%) ($P = 0.51$). The presence of intestinal metaplasia did not differ between groups (Table 2).

Virtually all biopsies had positive (intermediate or high) staining for MUC5AC in the surface and neck cells (Figure 1). Staining for MUC5AC was minimal in gland cells (Tables 3 and 4). Although no significant difference between groups was observed there was a trend towards

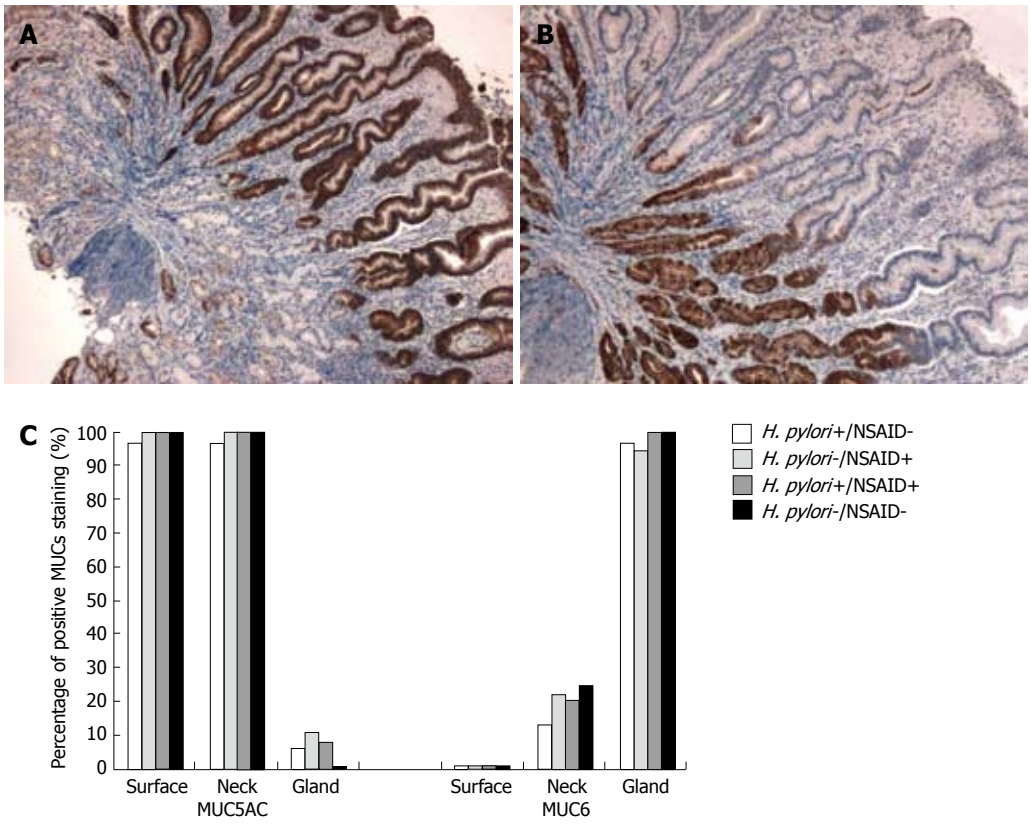

Figure 1 Mucin 5AC and mucin 6 expression in gastric ulcers. A: Immunohistochemical stain showing strong expression of mucin 5AC (MUC5AC) in surface and neck cells (× 10) of gastric mucosa from the ulcer edge of a patient with *Helicobacter pylori* (*H. pylori*)/non-steroidal anti-inflammatory drugs (NSAID)-negative gastric ulcer; B: Immunohistochemical stain showing strong expression of MUC6 in deep gland cells (× 10) of gastric mucosa from the ulcer edge of a patient with *H. pylori*/NSAID-negative gastric ulcer; C: Percentage of positive MUCs staining according to *H. pylori* and NSAID status. Positive stain defined as a stain extent and intensity score product ⩾ 2.

Table 5 Intraepithelial/mucosal T-cell populations (n = 20)

Group	CD4+/CD8+	CD8+/HPF	CD4+/HPF
H. pylori+/NSAID-	3.15	22.0 ± 4.4	69.6 ± 38.2[a]
H. pylori-/NSAID+	0.55	14.0 ± 9.4	8.4 ± 12.5
H. pylori+/NSAID+	2.99	26.6 ± 20.9	50.0 ± 25.2
H. pylori-/NSAID-	1.15	29.0 ± 11.4	38.0 ± 27.7

[a]P = 0.009 *vs H. pylori*-/NSAID+ group. *H. pylori*: *Helicobacter pylori*; NSAID: Non-steroidal anti-inflammatory drugs; HPF: High-power field.

decreased expression of MUC5AC in the deep glands of idiopathic ulcers (P = 0.09). Including for analysis only cells with high mucin expression (extent and intensity score products of 6 or 9), and separate analyses of staining extent and intensity, did not significantly alter the results. All but two biopsies stained positively (intermediate or high) for MUC6 in the gland cells (Figure 1), and staining was uniformly negative in the surface cells. Staining for MUC6 was variable in the neck cells. Again, including only cells with high mucin expression (extent and intensity score products of 6 or 9), and separate analyses of staining extent and intensity, did not significantly alter the results.

T-cell CD4/CD8 ratio was significantly lower in the NSAID-positive groups when compared to the *H. pylori*-positive groups (P = 0.009, Table 5). As expected, the Sydney inflammation score was significantly lower in the *H. pylori*-negative/NSAID-negative group than in the *H. pylori*-positive/NSAID-negative group (P = 0.017, Table 6).

DISCUSSION

Gastric secreted mucin expression has not been previously studied in the setting of the idiopathic ulcer. In the present study, the distribution of immunohistochemical staining for MUC5AC and MUC6 in the margins of *H. pylori*/NSAID-negative ulcers did not significantly differ from staining patterns in the margins of ulcers associated with *H. pylori* and/or NSAIDS.

H. pylori interacts with gastric mucins in various manners, in order to facilitate its colonization. It has been established that *H. pylori* disrupts the assembly of the mucin molecule *via* inhibition of galactosyltransferase[10,12]. Furthermore, *H. pylori* reduces gastric mucous viscosity by elevating pH through urease secretion, thereby enhancing its motility within gastric mucous[15]. Kobayashi *et al*[16] demonstrated that BabA and SabA adhesins on *H. pylori* bind to Lewis B blood group antigens on MUC5AC, facilitating colonization.

On the other hand, gastric mucins have antimicrobial properties which are directed against *H. pylori*. Kawakubo *et al*[11] demonstrated that unique O-glycans in MUC6 inhibit bacterial biosynthesis of cholesteryl-α-*D*-glucopyranoside,

Table 6 Inflammation score according to the Sydney system (n = 20)

Group	*H. pylori*	Atrophy	Intestinal metaplasia	Lymphocytes	PMN	Score
H. pylori+/NSAID-	2.20 ± 0.44	0.60 ± 0.54	0.40 ± 0.54	1.80 ± 0.83	1.20 ± 1.30	6.20 ± 2.48[a]
H. pylori-/NSAID+	0.00 ± 0.00	0.40 ± 0.54	0.60 ± 1.34	1.40 ± 0.89	0.20 ± 0.44	2.60 ± 2.19
H. pylori+/NSAID+	1.80 ± 1.09	0.80 ± 0.83	0.40 ± 0.89	2.00 ± 0.70	1.40 ± 0.89	6.40 ± 3.13
H. pylori-/NSAID-	0.00 ± 0.00	0.20 ± 0.44	0.20 ± 0.44	1.40 ± 0.54	0.40 ± 0.54	2.20 ± 1.64

[a]P = 0.017 *vs H. pylori*-/NSAID- group. PMN: Polymorphonuclear cells; *H. pylori*: *Helicobacter pylori*; NSAID: Non-steroidal anti-inflammatory drugs.

a major cell wall component. Lindén *et al*[17] suggest that mucins decorated with Le^b (the binding site for the *H. pylori* BabA adhesin) effectively bind *H. pylori*, thereby impairing its colonization of the mucosal surface. Similarly, in *Trichuris muris* infection MUC5AC is aberrantly expressed in the intestine and plays a key role in expulsion of the nematode[18]. Byrd *et al*[19] demonstrated that gastric biopsies from *H. pylori*-related gastritis patients frequently expressed the aberrant location of MUC6 staining in the surface foveolar cells. In the present study, cytoplasmic mucin staining of ulcers associated with *H. pylori* was unchanged. One reason for this discrepancy is the fact that the present study only included patients with gastric ulcers, and sampled gastric mucosa immediately adjacent to the edge of a gastric ulcer. These patients represent a distinct subgroup of patients with *H. pylori* infection, and our findings imply that alterations in secreted gastric mucins do not play a role in the pathogenesis of gastric ulcer in patients with *H. pylori* infection. This is supported by Marques *et al*[20] who found that although topographic expression of MUC5AC and MUC6 mucins was altered in *H. pylori*-related gastritis, the expression of these mucins was unchanged in mucosa adjacent to patients with *H. pylori*-related gastric ulcer.

An interesting finding in our study was that many patients with *H. pylori*/NSAID-negative ulcers had multiple comorbidities, were more often inpatients at the time of endoscopy, had fewer subacute presentations, and had a poorer survival. This concurs with Chan *et al*[21] who noted that three-quarters of patients with acutely bleeding *H. pylori*/NSAID-negative ulcers have significant comorbidity including major organ failure and malignancy. A large prospective study found that concomitant diseases and the absence of epigastric pain are independent risk factors for *H. pylori*/NSAID-negative ulcers in the duodenum[22]. A higher number of comorbidities were associated with increased ulcer size and depth, and more bleeding complications. Furthermore, our data are consistent with previous findings that idiopathic ulcer is an independent risk factor associated with long-term mortality[23,24].

In this study, *H. pylori*/NSAID-negative gastric ulcers were associated with underlying systemic disease, which could be severe. This association has been reported previously in idiopathic ulcers[24], and suggests the possible role of ischemic or non-specific inflammatory factors in their pathogenesis[25]. Despite the heterogeneity of possible pathogenic factors of these idiopathic *H. pylori*/NSAID-negative cases, our results indicate virtually no difference in qualitative MUC5AC and MUC6 staining in these ulcers compared with definite *H. pylori*-positive, NSAID-positive, and combined *H.pylori*/NSAID-positive gastric ulcers .

H. pylori-positive ulcer is associated with a high inflammation rate, thus groups 2 and 4 had significantly lower Sydney inflammation score than groups 1 and 3 (P = 0.017). In addition, we found a low ratio of T-cell CD4/CD8 in the groups negative for *H. pylori*; but when NSAID use was also negative the result did not reach significance. Similar findings were described by Strömberg *et al*[26]. In peptic ulcer patients positive for *H. pylori*, the number of intraepithelial T-cell CD4+ was higher than in patients with *H. pylori* infection but without ulcer or in healthy controls negative for *H. pylori*. Thus, *H. pylori* infection recruits CD4+ lymphocytes.

A limitation of our study is the retrospective nature of the data collection, which precluded elimination of false negative tests for *H. pylori* (probably due to proton pump inhibitor, bismuth or antibiotics), and cases of surreptitious or unreported NSAID use, which would result in misclassification of ulcers as *H. pylori*/NSAID-negative. This could only be overcome using a prospective study design, by performing multiple tests for *H. pylori* and assaying serum salicylate and plasma thromboxane, respectively.

In conclusion, patterns of mucin secretion in *H. pylori*/NSAID-negative ulcers need to be further studied in well-designed, prospective studies which minimize cross-contamination of groups. Idiopathic peptic ulcers are an increasingly encountered entity, with unique clinical and endoscopic features. Future efforts should focus on identifying genetic and epigenetic factors which regulate mucin secretion in this setting, as well as characterizing a potential role of the membrane-bound mucins and other mucosal protective factors.

COMMENTS

Background

In health, a mucin layer protects the stomach from the harmful effects of gastric acid and ulceration. The main causes of peptic ulcer disease are *Helicobacter pylori* (*H. pylori*) infection and aspirin or nonsteroidal anti-inflammatory drug (NSAID) therapy. However, up to 39% of peptic ulcer disease is idiopathic. Idiopathic ulcers are often associated with poorer outcomes.

Research frontiers

Alterations in secreted gastric mucins have been described in *H. pylori* infection and NSAID treatment, and may have a role in ulcer pathogenesis. This is the first study to look at gastric mucin secretion in idiopathic ulcers.

Innovations and breakthroughs

Mucin 5AC (MUC5AC) and MUC6 are equally expressed by *H. pylori*-induced,

NSAID-induced and idiopathic ulcers. The number of CD4 and CD8 lymphocytes is highest in *H. pylori*-induced ulcers reflecting a greater inflammatory component. Inflammation score is lowest in NSAID-associated ulcers.

Applications

Idiopathic peptic ulcer disease is more aggressive than peptic ulcer disease induced by *H. pylori* or NSAIDs. Elucidating the pathogenesis of idiopathic ulcers will lead the way to finding appropriate therapies.

Terminology

Mucin is the major component of gastric mucous and is synthesized and secreted by specialized cells. The two primary gastric mucin molecules are coined MUC5AC and MUC6; *H. pylori* is a ubiquitous bacterium which may cause gastric ulcer; NSAIDs include a range of over-the-counter medications commonly used for analgesia and rheumatic disease, and similarly cause gastric ulceration. Idiopathic ulcers are ones with no identifiable cause.

Peer review

The manuscript is interesting and has potential to enhance understanding on MUC5AC and MUC6 expression in gastric mucosa in *H. pylori*-, NSAID- and idiopathic-ulcers.

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BRIEF ARTICLE

What MELD score mandates use of entecavir for ACLF-HBV HBeAg-negative patients?

Ying Yan, Li Mai, Yu-Bao Zheng, Shao-Quan Zhang, Wen-Xiong Xu, Zhi-Liang Gao, Wei-Min Ke

Ying Yan, Li Mai, Yu-Bao Zheng, Shao-Quan Zhang, Wen-Xiong Xu, Zhi-Liang Gao, Wei-Min Ke, Department of Infectious Diseases, The Third Affiliated Hospital of Sun Yat-sen University, Guangzhou 510630, Guangdong Province, China
Author contributions: Yan Y and Mai L contributed equally to this work; Yan Y, Mai L and Ke WM designed the research; Yan Y, Mai L, Zheng YB, Zhang SQ, Xu WX and Gao ZL performed the research; Yan Y, Mai L, Zheng YB, Zhang SQ and Xu WX collected data; Yan Y and Mai L analyzed the data; Yan Y, Mai L and Ke WM wrote the paper.

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Correspondence to: Wei-Min Ke, Professor, Department of Infectious Diseases, The Third Affiliated Hospital of Sun Yat-sen University, Guangzhou 510630, Guangdong Province, China. kwm1999@163.com
Telephone: +86-20-85253172 Fax: +86-20-85253172
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Abstract

AIM: To investigate optimal timing for therapeutic efficacy of entecavir for acute-on-chronic hepatitis B liver failure (ACLF-HBV) in hepatitis B e antigen (HBeAg)-negative patients.

METHODS: A total of 109 inpatients with ACLF-HBV were recruited from the Department of Infectious Diseases of the Third Affiliated Hospital, Sun Yat-sen University from October 2007 to October 2010. Entecavir 0.5 mg/d was added to each patient's comprehensive therapeutic regimen. Patients were divided into three groups according to model for end-stage liver disease (MELD) score: high ($\geqslant$ 30, 20 males and 4 females, mean age 47.8 ± 13.5 years); intermediate (22-30, 49 males and 5 females, 45.9 ± 12.4 years); and low ($\leqslant$ 22, 28 males and 3 females, 43.4 ± 9.4 years). Statistical analysis were performed using SPSS 11.0 software. Data with normal distribution were expressed as mean ± SD and comparisons were made with Student's *t* tests. A value of $P < 0.05$ was considered statistically significant. Viral loads were related exponentially and logarithmic data were used for analysis.

RESULTS: For 24 patients with MELD score $\geqslant$ 30, treatment lasted 17.2 ± 16.5 d. Scores before and after treatment were significantly different (35.97 ± 4.87 and 40.48 ± 8.17, respectively, $t = -2.762$, $P = 0.011$); HBV DNA load was reduced (4.882 ± 1.847 copies $\log_{10}$/mL to 3.685 ± 1.436 copies $\log_{10}$/mL); and mortality rate was 95.83% (23/24). Of 54 patients with scores of 22-30, treatment lasted for 54.0 ± 43.2 d; scores before and after treatment were 25.87 ± 2.33 and 25.82 ± 13.92, respectively ($t = -0.030$, $P = 0.976$); HBV DNA load decreased from 6.308 ± 1.607 to 3.473 ± 2.097 copies $\log_{10}$/mL; and mortality was 51.85% (28/54). Of 31 patients with scores $\leqslant$ 22, treatment lasted for 66.1 ± 41.9 d; scores before and after treatment were 18.88 ± 2.44 and 12.39 ± 7.80, respectively, ($t = 4.860$, $P = 0.000$); HBV DNA load decreased from 5.841 ± 1.734 to 2.657 ± 1.154 copies $\log_{10}$/mL; and mortality was 3.23% (1/31).

CONCLUSION: For HBeAg-negative patients with ACLF-HBV, when entecavir was added to comprehensive therapy, a MELD score $\geqslant$ 30 predicted very poor prognosis due to fatal liver failure.

Key words: Acute-on-chronic hepatitis B liver failure; Hepatitis B e antigen negativity; Entecavir; Model for end-stage liver disease; Mortality

Peer reviewer: Tatsuya Ide, MD, PhD, Assistant Professor, Division of Gastroenterology, Department of Medicine, Kurume University School of Medicine, 67 Asahi-machi, Kurume 830-0011, Japan

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INTRODUCTION

Currently, there are only four commercially available nucleoside analogs for the treatment of hepatitis B virus (HBV). These include lamivudine, adefovir dipivoxil, entecavir and telbivudine; all efficient antiviral agents that can reduce the HBV DNA load to the lower limit of detection in 72%, 51%, 90% and 88% of hepatitis B e antigen (HBeAg)-negative chronic hepatitis B (CHB) patients, respectively, after 1 year of treatment[1-5]. However, these drugs seem to be ineffective for some patients with acute-on-chronic hepatitis B liver failure (ACLF-HBV), for whom even comprehensive therapy in combination with antiviral treatment cannot reduce a high mortality rate[6,7]. When compared with HBeAg-positive CHB patients, the proportion of HBeAg-negative CHB patients has been increasing during recent years, and the age of these patients is relatively advanced. Although the HBV-DNA load is relatively low, this form of hepatitis is active, prone to fluctuation, and positively correlated with the degree of inflammation and necrosis of the liver[8,9]. Furthermore, cirrhosis and hepatocellular carcinoma are more prevalent among these patients. In a previously reported study, 71.74% of patients with ACLF-HBV were negative for HBeAg[6]. Here, we evaluated the efficacy of entecavir, a potent antiviral nucleoside analog, in ACLF-HBV patients having different model for end-stage liver disease (MELD) scores, and suggest a new method for guiding the therapeutic decision-making process.

MATERIALS AND METHODS

Patients

A total of 109 inpatients with ACLF-HBV were recruited from the Department of Infectious Diseases of the Third Affiliated Hospital, Sun Yat-sen University from October 2007 to October 2010. Diagnosis was based on the Guidelines for Diagnosis of Liver Failure (2006)[10] and included the presence of two or more of the following: prothrombin time/international normalized ratio (PT-INR) $\geqslant$ 1.5; serum total bilirubin > 10 times the upper limit of normal; ascites; hepatic encephalopathy; decreased liver size; or the hepatorenal syndrome. The standard serum creatinine (Cr) was defined as the normal median (31.8-93.7 μmol/L), and MELD scores were $\geqslant$ 16.1. All patients were hepatitis B surface antigen (HBsAg)-positive, HBeAg-negative, hepatitis B e antibody (HBeAb)-positive and hepatitis B core antibody (HBcAb)-positive. There were 97 male (89.0%) and 12 female (11.0%) patients with a mean age of 45.61 ± 11.90 years (range: 25-73 years). Patients were divided into three groups according to MELD score: high ($\geqslant$ 30, 20 male and 4 female, mean age 47.8 ± 13.5 years); intermediate (22-30, 49 male and 5 female, 45.9 ± 12.4 years); and low ($\leqslant$ 22, 28 male and 3 female, 43.4 ± 9.4 years). There were no marked differences in age and sex among the three groups. Patients co-infected with hepatitis A, C or E virus or human immunodeficiency virus were excluded. None had been treated with regular pegylated interferon, nucleoside analogs, or thymosin. Entecavir 0.5 mg/d was added to each patient's comprehensive therapeutic regimen as the sole antiviral agent.

Detections

HBV DNA levels were determined by real-time polymerase chain reactions with commercial diagnostic kits (Da-an GeneCo., Guangzhou, China). The lower detection limit was 500 copies/mL. HBsAg, HBsAb, HBeAg, HBeAb and HBcAb were detected with an automatic rapid immunoassay system (AxSYM; Abbott, United States). Total bilirubin and Cr and were measured using an automatic biochemical analyzer (AU 640; Olympus, Japan); normal ranges were 4-17.1 μmol/L and 31.8-93.7 μmol/L, respectively. PT-INR = (prothrombin time/reference prothrombin time) ISI. Prothrombin time was measured using detection reagent STA-Neoplastine(r) CI PLUS with an automatic coagulometer (STA-R) (Diagnostica Stago, France). Sample collection, transportation, preservation and processing were performed according to the manufacturer's instructions.

Calculations of MELD scores

MELD score = 3.8 × $\log_e$ [serum bilirubin (μmol/L) × 0.058] + 11.2 × $\log_e$ (PT-INR) + 9.6 × $\log_e$ [serum Cr (μmol/L) × 0.011] + 6.4 × (0 or 1) (cholestatic or alcoholic cirrhosis: 0; other liver diseases: 1)[11].

Definitions of therapeutic efficacy

Survival: patients discharged with no evidence of liver failure; death: progression of disease leading to death in spite of comprehensive therapy and entecavir treatment.

Statistical analysis

Statistical analysis were performed using SPSS 11.0 software. Data with normal distribution were expressed as mean ± SD and comparisons were made with Student's *t* tests. A value of $P < 0.05$ was considered statistically significant. Viral loads were related exponentially and logarithmic data were used for analysis.

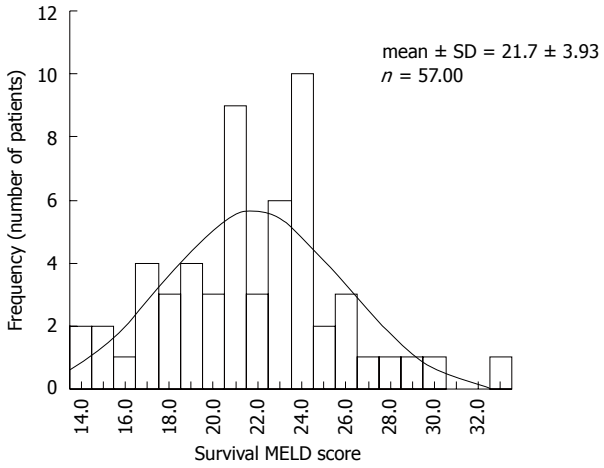
RESULTS

The mortality rate was > 95% in patients with MELD score > 30, and > 95% of patients survived with MELD

A

Statistics

Survival		
n	Valid	57
	Missing	14
mean		21.67867
median		21.54514
Percentiles	5	14.60103
	25	18.74029
	50	21.54514
	75	23.75528
	95	28.63190
	96	29.38532
	97	30.69348



B

Statistics

Death		
n	Valid	52
	Missing	19
mean		30.9545
median		29.1479
Percentiles	3	21.9790
	4	22.2608
	5	22.8505
	25	27.6117
	50	29.1479
	75	34.6216
	95	43.9692

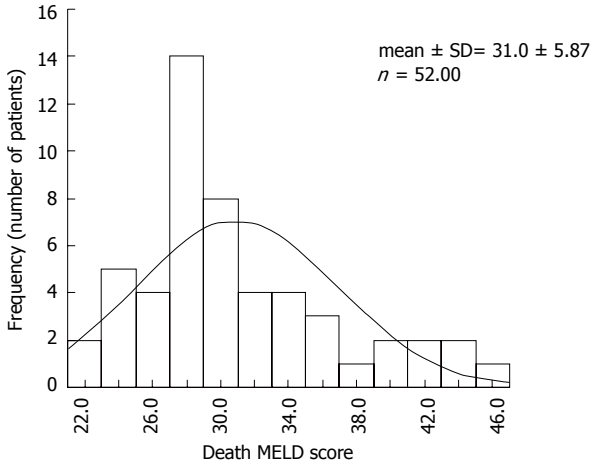


Figure 1 Frequency distribution of model for end-stage liver disease scores of patients receiving entecavir who survived (A) or succumbed (B). Death occurred in 95% of patients with MELD score > 28.63, 96% with score > 29.39 and 97% with score > 30.69; and in 5% with score < 22.85, 4% with score < 22.26, and 3% with score < 21.97. The mortality rate was > 95% in patients with MELD score > 30, and > 95% patients survived with MELD score < 22. MELD: Model for end-stage liver disease.

score < 22. Frequency distribution of MELD scores of patients receiving entecavir who survived or succumbed is described in Figure 1.

Of the 24 patients with high MELD scores (≥ 30), 23 (95.83%) died. Antiviral treatment was administered for 17.2 ± 16.5 d. Comparing measurements taken before antiviral treatment and before death, there were no marked differences in HBV DNA load, total bilirubin, INR and prothrombin activity (PTA), but Cr and MELD scores increased significantly (P = 0.003 and 0.011, respectively) (Table 1).

Of the 54 patients with intermediate MELD scores, 28 (51.85%) died and 26 survived. Antiviral treatment was administered for 54.0 ± 43.2 d. The MELD scores before and after antiviral treatment were not significantly different (25.87 ± 2.33 and 25.82 ± 13.92, respectively; t = -0.030, P = 0.976), but the HBV DNA load decreased significantly, by 2.918 ± 1.811 copies $\log_{10}$/mL (P = 0.000). Liver function results and outcomes are presented in Table 1.

There were 31 patients with low MELD scores; one (3.23%) died and 30 survived. Antiviral treatment was administered for 66.1 ± 41.9 d. Total bilirubin decreased and PTA increased significantly during antiviral treatment (P < 0.05), and decreases in MELD score (18.88 ± 2.44 to 12.39 ± 7.80; t = 4.860, P = 0.000) and HBV DNA levels (by 3.185 ± 1.740 copies log10/mL, P = 0.000) were also significant (Table 1).

DISCUSSION

Providing comprehensive treatment is the main therapeutic strategy for patients with ACLF; when possible other treatment options include use of bioartificial liver support systems and liver transplantation. Comprehensive treatment can only prolong survival for the fraction of patients having a potential for liver regeneration. When antiviral treatment is added, prolonged survival times can be achieved at low cost. It remains controversial whether comprehensive therapy with bioartificial liver support systems is translated into a survival benefit. Liver transplantation is usually limited by high cost, insufficient donors, and unclear effects on long-term survival. In recent years, treatment of liver failure with stem cell transplantation has achieved favorable outcomes in preclinical studies, but this strategy has not been approved and its

Table 1 Models for end-stage liver disease score group: Liver function, hepatitis B virus DNA load, length of treatment and mortality rate

n = 24	Total bilirubin (μmol/L)	INR	Prothrombin activity (%)	Blood creatinine (μmol/L)	MELD score	HBV DNA loads (copies log10/mL)	Treatment course (d)	Mortality (%)
High model (*n* = 24)								
Before treatment	563.8 ± 163.0	4.14 ± 1.05	21.2 ± 5.8	124.0 ± 113.1	35.97 ± 4.87	4.882 ± 1.847	17.2 ± 16.5	95.83 (23/24)
Before death	615.3 ± 216.4	4.67 ± 1.71	20.0 ± 8.1	179.1 ± 129.1[b]	40.48 ± 8.17	3.685 ± 1.436		
t value	-1.742	-1.699	0.738	-3.340	-2.762	2.590		
P value	0.095	0.103	0.468	0.003	0.011	0.027		
Intermediate model (*n* = 54)								
Before treatment	444.5 ± 160.3	2.56 ± 0.71	35.6 ± 12.1	70.1 ± 15.8	25.87 ± 2.33	6.308 ± 1.607	54.0 ± 43.2	51.85 (28/54)
After treatment	409.5 ± 357.7	3.23 ± 2.26	38.4 ± 24.1	93.4 ± 75.8	25.82 ± 13.92	3.473 ± 2.097[b]		
t value	0.667	-2.637	-1.132	-2.223	-0.030	8.826		
P value	0.508	0.011	0.263	0.031	0.976	0.000		
Low model (*n* = 31)								
Before treatment	351.9 ± 114.8	1.59 ± 0.37	56.8 ± 14.1	62.5 ± 11.1	18.88 ± 2.44	5.741 ± 1.734	66.1 ± 41.9	3.23 (1/31)
Recovery stage	102.8 ± 144.4	1.55 ± 0.64	63.5 ± 18.2	67.4 ± 52.6	12.39 ± 7.80	2.657 ± 1.154		
t value	7.065	0.411	-2.468	-0.526	4.860	9.332		
P value	0.000	0.684	0.020	0.603	0.000	0.000		

Before death meaning: laboratory findings before articulo mortis. [b]$P < 0.01$ *vs* before treatment. MELD: Model for end-stage liver disease; HBV: Hepatitis B virus; INR: International normalized ratio.

therapeutic efficacy remains to be confirmed. At present, treatment with nucleoside analogs has been applied in the treatment of ACLF-HBV and has been confirmed effective, but not all patients can survive[12-16]. When is the right time to add nucleoside analogs for treatment of ACLF-HBV patients? Therefore, it is imperative to investigate the therapeutic efficacy of comprehensive therapy in combination with antiviral treatment.

For HBeAg-negative and -positive CHB patients, there are differences in indication for treatment with nucleoside analogs, their therapeutic efficacy, and criteria for discontinuation of treatment. In the natural course of CHB, the HBV viral load significantly increases once the host immune function is compromised, which may induce acute cellular immune responses, resulting in ACLF. Therefore, maximizing long-term inhibition or elimination of HBV DNA may prevent the occurrence of ACLF in HBeAg-negative patients[17]. Nucleoside analogs can rapidly inhibit the replication of HBV and minimize propagation of the virus between hepatocytes. In addition, antiviral treatment can reduce target antigens on hepatocytes, thereby reducing attacks by cytotoxic T cells, thus attenuating the extent of damage and subsequent necrosis.

Entecavir is a cyclopentyl guanosine analog that can significantly inhibit HBV DNA polymerase, which then suppresses initiation of DNA replication and extension, resulting in reduction of viral load[18-20]. Among four commercially available nucleoside analogs, entecavir is the most potent antiviral drug, and it has the lowest frequency of resistance (1% after 4 years)[21,22]. The US Guidelines for Diagnosis and Treatment of Chronic Hepatitis B recommends entecavir as the first-line antiviral drug for treatment of patients with HBV-related liver failure[23]. Therefore, the present study aimed to investigate the efficacy of entecavir in combination with comprehensive therapy of ACLF-HBV, according to MELD score.

In 2003, Malinchoc *et al*[11] analyzed clinical information of 231 patients undergoing transjugular intrahepatic portosystemic stent shunt procedures. They applied Cox proportional hazards regression to establish the MELD score, which was then used to evaluate the risk of death among patients with liver diseases of any cause, achieving favorable reliability. A 2002 study revealed that MELD scores were given priority in making the decision to perform liver transplantation[24]. In addition, MELD score is also a sensitive indicator of the severity of liver failure.

In the present study, mean scores in the high MELD score group increased significantly before death, and mortality was 95.83% (23/24). Therapeutic failure in this group may be attributed to the fact that suppression of viral replication requires a relative long period of time; a previous study has determined that treatment with entecavir can significantly reduce the viral load within 4 wk[25]. However, treatment in this group lasted on average only 17.2 d, in the setting of fatal liver failure. In addition, ACLF pathogenesis is complex; the rapid increase in HBV replication is only one factor contributing to liver failure. Once liver failure is initiated, additional antiviral treatment is not adequate to prevent progression.

No significant differences in scores were observed before and after antiviral treatment in the intermediate MELD score group, which may account for the difference in outcome [48.15% (26/54) recovered and 51.85% (28/54) died]. Death in this group may be related to late-stage ACLF or to deteriorating liver function in the early or intermediate stages. In this CHB condition, massive liver necrosis may occur from induction of potent cellular immune responses; as a result, antiviral treatment may not be effective. Survival of 48.15% (26/54) of these patients may be attributed to the potential for liver regeneration, even if several parameters suggest liver failure. In this group, the course of treatment was 54.0 d, so HBV DNA replication was significantly suppressed, and fur-

ther liver necrosis was prevented.

In the low MELD score group, scores were significantly decreased by antiviral treatment, and the mortality was 3.23% (1/31). The majority of patients recovered, possibly because they had early-stage ACLF, a low risk for development of cellular immune responses, and the potential for compensatory liver regeneration. The mean length of treatment was 66.1 d; enough time for entecavir to accomplish an antiviral effect, which, together with effective liver regeneration, promotes recovery from liver failure.

We suggest that timely administration of an antiviral agent may improve prognosis for HBeAg-negative ACLF patients, and that prognosis can be predicted by the MELD score. When the score is $\leqslant$ 22, most patients may survive if given comprehensive therapy in combination with antiviral treatment. When the MELD is 22-30, survival, if given antiviral treatment, will depend on the severity of liver failure, the developmental trend of the disease, and whether comprehensive therapeutic interventions provide enough time for antiviral drugs to exert their effects. When the MELD score is $\geqslant$ 30, the vast majority of patients will not recover with comprehensive plus antiviral therapy. These patients are at high risk for death and liver transplantation should be considered.

COMMENTS

Background

In the natural course of chronic hepatitis B, the hepatitis B virus (HBV) viral load significantly increases once the host immune function is compromised, which may induce acute cellular immune responses, resulting in acute-on-chronic liver failure (ACLF). Therefore, maximizing long-term inhibition or elimination of HBV DNA may prevent the occurrence of ACLF in hepatitis B e antigen (HBeAg)-negative patients.

Research frontiers

In previous studies, treatment with nucleoside analogs has been used for acute-on-chronic hepatitis B liver failure (ACLF-HBV) and has been confirmed as effective. When is the right time to add nucleoside analogs to treatment of ACLF-HBV patients? The present study investigated the optimal timing, according to model for end-stage liver disease (MELD) score, for therapeutic efficacy of entecavir for ACLF-HBV in HBeAg-negative patients.

Innovations and breakthroughs

For HBeAg-negative patients with ACLF-HBV, when entecavir was added to comprehensive therapy, patients with different MELD scores may have a different outcome; a MELD score $\geqslant$ 30 predicted very poor prognosis due to fatal liver failure.

Peer review

This issue is of great interest and is offering a new method of deciding the optimal timing for therapeutic efficacy of entecavir to treat ACLF-HBV in HBeAg-negative patients according to MELD score.

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BRIEF ARTICLE

Side population cells isolated from KATO Ⅲ human gastric cancer cell line have cancer stem cell-like characteristics

Jun-Jun She, Peng-Ge Zhang, Xuan Wang, Xiang-Ming Che, Zi-Ming Wang

Jun-Jun She, Xiang-Ming Che, Department of General Surgery, First Affiliated Hospital of Medical College, Xi'an Jiaotong University, Xi'an 710061, Shaanxi Province, China
Peng-Ge Zhang, Department of Mathematics, Xidian University, Xi'an 710061, Shaanxi Province, China
Xuan Wang, Department of Endocrinology, First Affiliated Hospital of Medical College, Xi'an Jiaotong University, Xi'an 710061, Shaanxi Province, China
Zi-Ming Wang, Department of Urology, Second Affiliated Hospital of Medical College, Xi'an Jiaotong University, Xi'an 710004, Shaanxi Province, China
Author contributions: She JJ, Che XM, Wang ZM made substantial contributions to conception and design; She JJ performed the majority experiment and drafted the article, revised it critically for important intellectual content; Wang X performed partial experiments and revised the manuscript; Zhang PG did the data analysis; all authors approved the version to be published.
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Correspondence to: Jun-Jun She, PhD, Department of General Surgery, First Affiliated Hospital of Medical College, Xi'an Jiaotong University, Xi'an 710061, Shaanxi Province, China. sjuns@sina.com
Telephone: +86-29-85323870 Fax: +86-29-88202132
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Abstract

AIM: To investigate whether the side population (SP) cells possess cancer stem cell-like characteristics *in vitro* and the role of SP cells in tumorigenic process in gastric cancer.

METHODS: We analyzed the presence of SP cells in different human gastric carcinoma cell lines, and then isolated and identified the SP cells from the KATO Ⅲ human gastric cancer cell line by flow cytometry. The clonogenic ability and self-renewal were evaluated by clone and sphere formation assays. The related genes were determined by reverse transcription polymerase chain reaction. To compare tumorigenic ability, SP and non-side population (NSP) cells from the KATO Ⅲ human gastric cancer cell line were subcutaneously injected into nude mice.

RESULTS: SP cells from the total population accounted for 0.57% in KATO Ⅲ, 1.04% in Hs-746T, and 0.02% in AGS (CRL-1739). SP cells could grow clonally and have self-renewal capability in conditioned media. The expression of *ABCG2*, *MDRI*, *Bmi-1* and *Oct-4* was different between SP and NSP cells. However, there was no apparent difference between SP and NSP cells when they were injected into nude mice.

CONCLUSION: SP cells have some cancer stem cell-like characteristics *in vitro* and can be used for studying the tumorigenic process in gastric cancer.

Key words: Side population; Cancer stem cells; Self-renewal; Gastric cancer; KATO Ⅲ

Peer reviewers: Kazuaki Takabe, MD, PhD, Assistant Professor of Surgery and Assistant Professor of Biochemistry and Molecular Biology, Surgical Oncology, Virginia Commonwealth University Massey Cancer Center, Medical College of Virginia, Virginia Commonwealth University, PO Box 980011, Richmond, VA 23298-0011, United States; Hikaru Nagahara MD, PhD, Professor, Aoyama Hospital, Tokyo Women's Medical University, 2-7-13 Kita-Aoyama, Minatoku, Tokyo 107-0061, Japan

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INTRODUCTION

Cancer stem cells (CSC) have the characteristics of longevity, self-renewal and proliferation. It is believed that cancer stem cells are responsible for the heterogeneity and relapse of certain cancers. Flow cytometric analysis of cell surface antigens has served as the main tool to characterize CSC. CSC populations have already been identified in several solid tumors, including breast cancer[1], brain tumor[2], prostate cancer[3], melanoma[4], retinoblastoma[5], lung cancer[6], and colon cancer[7]. CSC often expresses the multi-drug resistant 1 (MDR1) or adenosine-triphosphate (ATP)-binding cassette (ABC) transporter genes, which help promote chemoresistance, a phenotype of side population (SP) cells[8].

SP cells can efflux the DNA-binding dye Hoechst33342 through an ABC membrane transporter. SP cells were initially investigated as part of the hematopoietic stem cells, and harbored stem cell-like characteristics[9]. Although conflicting results have been recently reported[10,11], most studies that investigated cell lines and tumors such as Cal-51[12], human breast carcinoma (MCF7)[13], human glioma (U373)[14], mouse ovarian carcinoma (MOVCAR 7)[15], human nasopharyngeal carcinoma (CNE-2)[16], Huh7 and PLC/PRF/5 hepatocellular carcinoma[15,16], 4T1 and NXS2 murine carcinoma[17] showed that these cell lines or tissues possessed SP cells, which were described to have stem cell-like fractions. Most studies suggested that SP cell analysis can be used to identify cancer stem cell populations[18].

Gastric cancer is the second most common cancer in the world. A total of 21 000 new cases of gastric cancer were diagnosed in the United States in 2010 with a projected five-year mortality rate exceeding 65%[19]. Aggressive surgery followed by chemotherapy resulted in a clinical response of 20%-35%. However, a majority of patients relapse and become drug-resistant. Various types of ABC transporters, especially ABC transporter genes 2 (*ABCG2*), contribute to drug resistance in cancers including gastric cancer, by pumping chemotherapeutic drugs out of cancer cells[20]. Therefore, SP cells could be used as a therapeutic target and for preventing relapse and drug-resistance in gastric cancer as well.

In this study, we analyzed the percentage of SP and non-side population (NSP) cells from several gastric cancer cell lines. By doing so, we sorted SP and NSP cells separately from the KATO Ⅲ cell lines and estimated whether the SP cell fraction possessed cancer stem cell-like characteristics *in vitro*.

MATERIALS AND METHODS

SP and NSP presentation

The human gastric cancer cell lines including KATO Ⅲ (ATCC NO: HTB-103), Hs-746T (HTB-135) and AGS (CRL-1739) were procured from American Type Culture Collection. KATO Ⅲ and Hs-746T were grown in DMEM supplemented with 100 mL/L fetal bovine serum (FBS) and penicillin/streptomycin at 37 ℃ in a humidified atmosphere with 50 mL/L CO_2. AGS was grown in RPMI-1640 supplemented with 100 mL/L FBS and penicillin/streptomycin at 37 ℃ in a humidified atmosphere with 50 mL/L CO_2. To analyze SP and NSP cell fractions, the cells were removed from their dishes with 2.5 g/L trypsin and 0.5 g/L ethylenediaminetetraacetic acid, centrifuged, washed with PBS and resuspended at 37 ℃ in Hank's balanced salt solution (HBSS) containing 20ml/L FBS. Cells (1×10^6) were labeled in HBSS with 5.0 mg/L Hoechst33342 dye (Sigma, St. Louis, MO) either alone or in combination with 50 mol/L verapamil (Sigma, St. Louis, MO) at 37 ℃ for 90 min. After washing three times with PBS, the cells were resuspended in HBSS containing 20 mL/L FBS and 1mmol/L 4-(2-hydroxyethyl)-1-piperazine ethanesulphonic acid (HEPES), passed through a 40-μm mesh filter, then maintained at 4 ℃ until flow cytometric analysis. Then, 1×10^6 viable cells were analyzed and sorted using a FACS Vantage SE Cell Sorter (BD Biosciences, CA). The Hoechst dye was excited with a UV laser and its fluorescence was measured with both 675/20 (Hoechst Red) and 424/44 filters (Hoechst Blue). The analysis was repeated three times.

Clone formation assays

Following melting 10 g/L agar (DNA grade) in the microwave and warming 2 × Dulbecco's modified eagle medium (DMEM) supplemented with 200 mL/L FBS to 40 ℃ in a water bath, equal volumes of the two solutions were mixed to yield a new solution of 5 g/L agar, 1 × DMEM and 100 mL/L FBS. Next, 1.0 mL of mixed solution was added to each well of a 6-well plate to form the base agar. Then, 7 g/L agar (DNA grade) was melted in the microwave and cooled to 40 ℃ in a water bath; similarly, 2 × DMEM and 200 mL/L FBS were warmed to the same temperature. Freshly sorted SP and NSP cells from KATO Ⅲ were passed through a 40-μm filter to provide a single cell suspension and were counted. Three mL DMEM, 1.5 mL 2 × DMEM containing 200 mL/L FBS and 1.5 mL agar including 400 cells were then mixed together, and a 1.5 mL cell suspension of this solution was placed into each well of a 6-well plate as the top agar. Finally, 100 cells were seeded into each well, and were incubated at 37 ℃ in a humidified incubator for 2-3 wk. Colonies were either left unstained, or were stained with 5 g/L MTT (Sigma, St. Louis, MO) for no more than one hour, and counted under a dissecting microscope. The procedure was repeated three times. After SP cells were seeded in soft agar assays, colonies containing more than 50 cells (primary colony) were removed from soft agar with sterile Pasteur pipettes, treated with trypsin and mechanically dissociated into single cells. Then, 100 cells were seeded into each well at 37 ℃ in a humidified incu-

bator for another 2-3 wk. Colonies (secondary colony) were stained with 5 g/L MTT for no more than one hour, and counted under a dissecting microscope. The procedure was repeated three times.

Lentivirus infection

Plasmid DNA was kept at a 3:3:1 ratio [Vector (EGFP): Δ8.91: VSV-G] according to the lipofectamine protocol (Invitrogen). Next, 2.5 μg plamid DNA was diluted in 500 μL serum-free DMEM for each well, and 2.5 μL Mix PLUS reagent and 6.25 μL Lipofectamine™ LTX reagent were added to the diluted DNA and incubated for 30 min at room temperature. Then, approximately 500 μL of the DNA-Lipofectamine™ LTX complex was added to each well containing 293FT cells, which were 40%-50% confluent. The cells were incubated at 37 ℃ in a CO_2 incubator for six hours and the medium was changed for transfected cells. After 24 h, the media from 293FT cells was collected, and 8 mg/L polybrene was added and transferred to target cells. The transfer of media from 293 FT to KATO Ⅲ cells was repeated after 24 h and 48 h.

Sphere formation assays

To test sphere formation in suspension, sorted SP and NSP cells from KATO Ⅲ were passed through a 40-μm filter to provide a single cell suspension. Next, 4 mL medium containing 100 cells were added to the 6-well plates with serum-free media including F12 with EGF (10 μg/L), insulin (20 mg/L) and basic fibroblast growth factor (bFGF) (10 μg/L). Aliquots of epidermal growth factor (EGF), insulin and bFGF were added twice a week. After 10-14 d, plates were visually assayed for the formation of floating spheres. To assess the ability of primary spheres to form secondary spheres, colonies containing more than 30 cells were collected by centrifugation and trypsinized to yield single cells. After passing through a 40-μm filter, 4 mL serum-free media containing 100 cells were added to each 6-well plate and were cultured 10-14 d for secondary sphere formation. Spheres were counted under a dissecting microscope.

Gene expression detection

1×10^5 SP and NSP cells from the KATO Ⅲ total population were collected in a separate centrifuge tube with 350 μL RLT buffer containing 10 g/L 2-mercaptoethanol, total RNA was extracted from these cells using a RNeasy Mini Kit (Qiagen, CA) according to the protocol provided by the manufacturer. RNA was transcribed into cDNA using the SuperScript First-Strand Synthesis System (Invitrogen, CA). RT-PCR was performed using a SuperScript One-Step kit (Invitrogen, CA). The primers were as follows: Glyceraldehyde-3-phosphate dehydrogenase, 5-CTG CAC CAC CAA CTG CTT AG-3 and 5-AGG TCC ACC ACT GAC ACG TT-3; *ABCG2*, 5-GGG TTC TCT TCT TCC TGA CGA CC-3 and 5-TGG TTG TGA GAT TGA CCA ACA GAC C-3; *MDR1*, 5-GCC TGG CAG CTG GAA GAC AAA TAC-3 and 5-ATG GCC AAA ATC ACA AGG GTT AGC-3; B-cell-specific Moloney murine leukemia virus insertion site 1 (*Bmi-1*), 5-AGC AGA AAT GCA TCG AAC AA-3 and 5-CCT AAC CAG ATG AAG TTG CTG A-3; Octamer-binding transcription factor 4 (*Oct-4*), 5-GAG AAT TTG TTC CTG CAG TGC-3 and 5-GTT CCC AAT TCC TTC CTT AGT G-3. The PCR products were separated by electrophoresis on a 20 g/L agarose gel.

Tumor formation assays

Sorted SP and NSP cells from KATO Ⅲ were resuspended in 50 μL HBSS, ranging in density from 10^4 cells to 10^3 cells, and then mixed with 50 μL Matrigel (Becton Dickinson, NJ) to prevent injected cell dispersion and loss. Then, cells were subcutaneously injected into 6- or 7-wk old nude mice on the day of sorting. Nude mice were obtained from the Animal Institute of the Xi'an Jiaotong University, China (XJTU). All experiments were approved by the Animal Care Committee of XJTU. Mice were monitored every day to assess tumor formation for 6-8 wk after transplantation.

Statistical analysis

All values were expressed as mean ± SD. Any significant difference among mean values was further evaluated by the Student's *t* test.

RESULTS

Identification of SP in gastric cancer cell lines

SP was identified through their fluorescence profile in a dual-wavelength analysis by flow cytometry (Hoechst red 675/20; Hoechst blue 424/44), and were shown as a characteristic tail separated from the complete population.

The percentage of SP cells in human gastric cancer cell lines was 0.57% in KATO Ⅲ, 1.04% in Hs-746T, and 0.02% in AGS (Figure 1). SP cells decreased in number following treatment with verapamil, an inhibitor of the ABC transporter. To further investigate the function of SP and NSP cells in these gastric cancer cell lines, *in vitro* and *in vivo* tumorigenicity was studied mainly among KATO Ⅲ cells.

Clone formation

The clonogenic ability of SP and NSP cells were examined when seeded as single cells. The clonogenic efficiency of SP cells was significantly higher than NSP cells in soft agar assays (Figure 2A and B). Moreover, most SP cells could divide into colonies of more than 50 cells in soft agar after three weeks. There was no apparent difference between NSP cells and the complete population. The self-renewal ability of SP cells was also examined in serial soft agar assays. There was an increase in clonogenic efficiency from primary to secondary colonies. Moreover, secondary colonies were similar in size to the primary colonies, thus demonstrating that SP cells can maintain and expand themselves in serial soft agar assays.

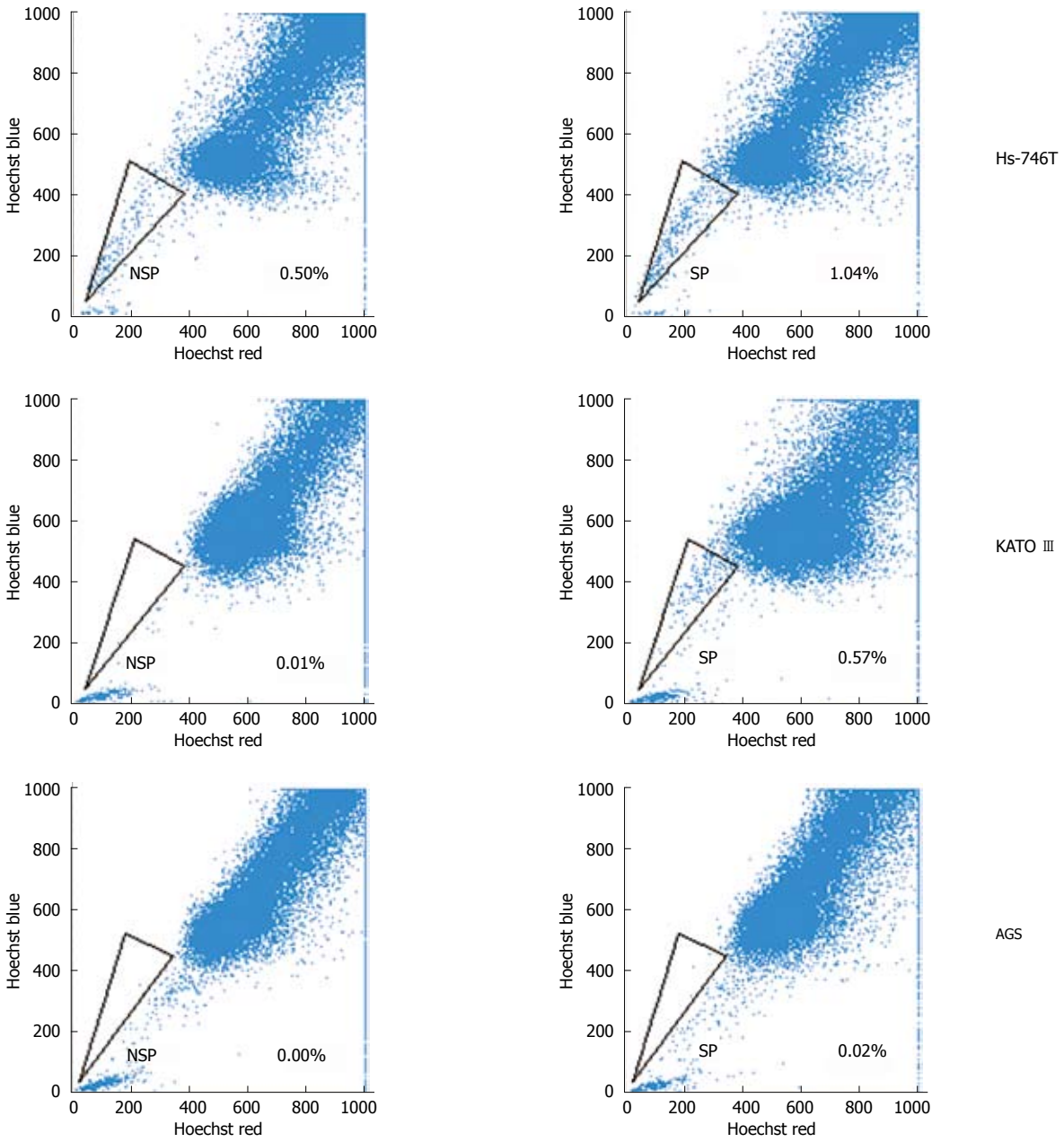

Figure 1 Side population and non-side population cells from several human gastric cancer cell lines were analyzed through uptake of the DNA binding dye Hoechst33342 with or without the presence of verapamil. NSP: Non-side population; SP: Side population.

To ascertain whether the colony comes from a single cell, KATO Ⅲ was infected with enhanced green fluorescent protein-expressing (EGFP) lentivirus and equal numbers of KATO Ⅲ-GFP and KATO Ⅲ cells were seeded from primary to second passage into serial soft agar assays. It was revealed that KATO Ⅲ-GFP cells expressed strong green fluorescence and showed a similar growth rate compared to non-infected KATO Ⅲ. Moreover, there were no mixed colonies (Figure 2C), indicating that the colonies came from single cells.

Sphere formation

The ability of SP and NSP cells to generate spherical clones and self-renewal was evaluated by sphere formation assays (Figure 3A). It was difficult to observe floating spheres from NSP cells (Figure 3B). SP cells started to form floating spheres after 2-3 d of seeding, and became primary spheres of more than 30 cells after 10-14 d (Figure 3C). The secondary sphere occurred slightly more quickly and frequently than the primary spheres, suggesting that SP cells have self-renewal ability.

Gene expression

First, the expression of ABC transporters, including *ABCG2* and *MDR1*, was observed, which was significantly higher in SP than in NSP cells (Figure 4). To further

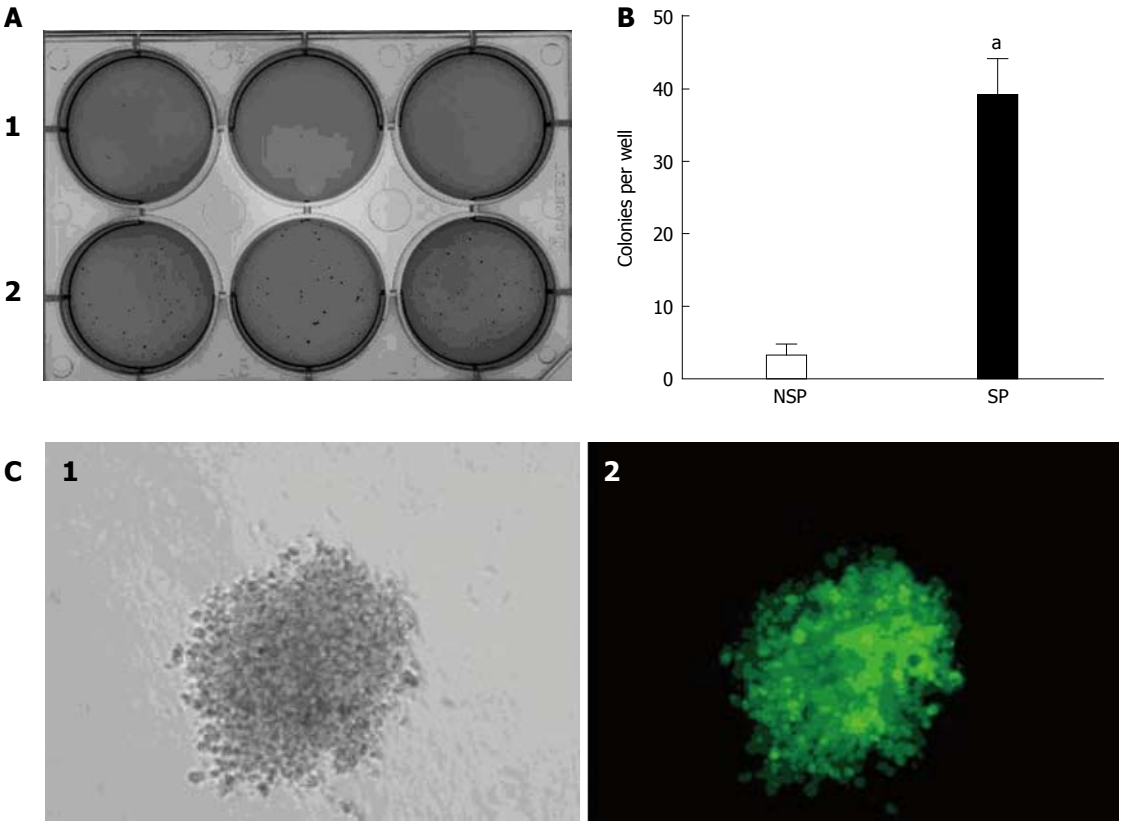

Figure 2 Soft agar assays and fluorescence for KATO Ⅲ-green fluorescent protein cells. A: Side population (SP) cells (A-2) were more clonogenic than non-side population (NSP) cells (A-1); B: The assay was repeated 3 times, and the column diagram indicated that there was a statistically significant difference in clonogenic efficiency between SP and NSP cells ($^aP < 0.05$ *vs* NSP cells); C: KATO Ⅲ-green fluorescent protein (GFP) cells expressed strong green fluorescence in regular culture, with no mixed colonies resulting from a single KATO Ⅲ-GFP cell. C-1 was observed under white light, while C-2 was observed under fluorescent light.

determine whether SP cells have stem cell-like characteristics, SP and NSP cells were examined for their expression of stem cell markers. The results demonstrated that the expression of *Oct-4* and *Bmi-1* in SP cells was higher than in NSP cells (Figure 4), suggesting that SP cells had some of the characteristics of undifferentiated stem cells.

Tumor formation

To compare tumorigenic ability, SP and NSP cells from the KATO Ⅲ human gastric cancer cell line were subcutaneously injected into the nude mice. Each mouse was individually injected with different amounts of SP and NSP cells. Six weeks after injection, although tumor diameter was smaller in NSP than in SP cells, four out of six mice could form tumors when injected either with SP or NSP cells (Table 1). All remaining mice could form tumors after eight weeks.

DISCUSSION

To our knowledge, there has been no report identifying SP cells from the gastric cancer cell lines. As such, in this paper we analyzed SP and NSP cells in the KATO Ⅲ, Hs-746T and AGS human gastric cancer cell lines. In the KATO Ⅲ and Hs-746T cell lines, which have a high tumorigenic ability, SP cells accounted for 0.57% and 1.04% of the total population, moreover, they occupied 0.02% in the high tumorigenic AGS cell line. Therefore, it is challenging to draw a conclusion from the percentage of SP cells in different cell lines to compare their tumorigenic ability. These data are consistent with the C6 glioma cell line, which was mainly composed of cancer stem cells, although many of these cells were neither CD133+ nor a side population[21]. Further studies are therefore needed to compare the relationship between SP prevalence and tumorigenicity.

Single cells that grow anchorage-independently in soft agar are known to be malignant. Thus, cell lines should contain those cells which could form colonies and those that did not form colonies in soft agar assays. As such, soft agar assays can be used to investigate this tumorigenic ability *in vitro*. By seeding the mixed cells of KATO Ⅲ-GFP and KATO Ⅲ into soft agar, we could conclusively determine that colonies came from single cells. The clonogenic ability of SP cells isolated from KATO Ⅲ was higher than that of NSP cells in soft agar and sphere forming assays. This is in agreement with the reports for the MCF7[13] and ARO[11] cell lines. In original animal transplant experiments, SP of KATO Ⅲ were injected into nude mice subcutaneously, ranging in density from 10^4 to 10^2 cells. The tumor forms in mice by injecting 10^4 (2 out of 2) and 10^3 cells (2 out of 2) SP in 6 wk. The other mice did not form tumor by injecting 5×10^2 (0 out of 2) and 1×10^2 cells (0 out of 2) SP until week

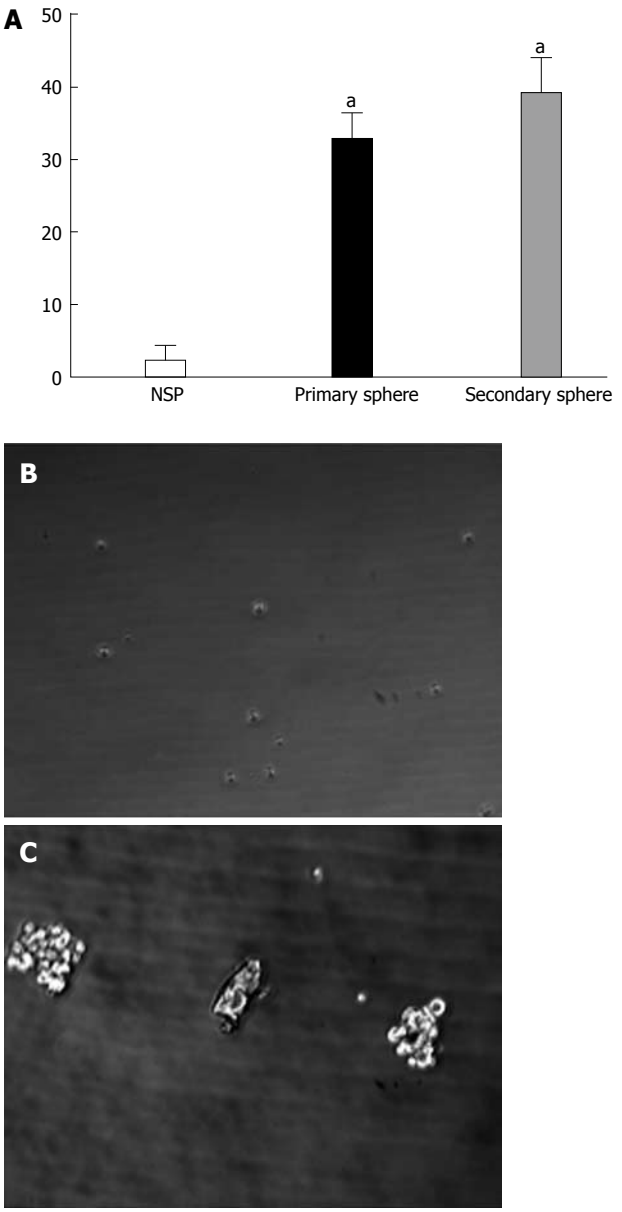

Figure 3 Serial sphere assays. A: The assay was repeated 3 times, and the column diagram indicated a statistically significant difference in clonogenic efficiency between non-side population (NSP) and side population (SP) cells in primary and secondary sphere assays ($^aP < 0.05$ *vs* NSP cells); however, there was no significant difference between primary and secondary sphere assays; B: Sphere assay for NSP cells; C: Sphere assay for SP cells.

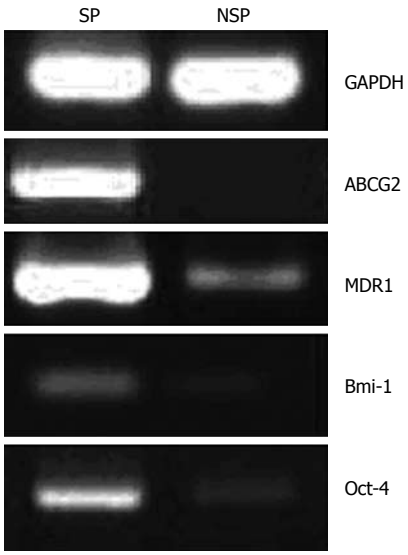

Figure 4 Glyceraldehyde-3-phosphate dehydrogenase, adenosine-triphosphate-binding cassette sub-family G member 2, multidrug resistance protein 1, B-cell-specific Moloney murine leukemia virus insertion site 1 and octamer-binding transcription factor 4 RNA expression of side population and non-side population cells isolated from KATO Ⅲ by reverse transcription polymerase chain reaction. SP: Side population; NSP: Non-side population; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; ABCG2: Adenosine-triphosphate-binding cassette sub-family G member 2; MDR1: Multidrug resistance protein 1; Bmi-1: B-cell-specific Moloney murine leukemia virus insertion site 1; Oct-4: Octamer-binding transcription factor 4.

Table 1 Tumor diameters in side population and non-side population cells sorted from the KATO Ⅲ cell line in nude mice after 6 wk

Mouse	Cell	Cell dose	Tumor diameter (cm)
A	SP	1×10^4	2.0
		1×10^3	1.0
	NSP	1×10^4	2.0
		1×10^3	0.5
B	SP	1×10^4	2.4
		1×10^3	1.3
	NSP	1×10^4	2.0
		1×10^3	Not tested
C	SP	1×10^4	2.0
		1×10^3	1.1
	NSP	1×10^4	2.0
		1×10^3	0.9
D	SP	1×10^4	2.2
		1×10^3	1.2
	NSP	1×10^4	1.9
		1×10^3	1.0
E	SP	1×10^4	1.8
		1×10^3	1.2
	NSP	1×10^4	1.8
		1×10^3	Not tested
F	SP	1×10^4	2.0
		1×10^3	1.0
	NSP	1×10^4	1.8
		1×10^3	1.0

SP: Side population; NSP: Non-side population.

10. In tumor formation assays, SP and NSP of KATO Ⅲ ranging from 10^4 to 10^3 cells were injected. In our study, 12 injection sites for SP cells could form tumors in six weeks. However, 12 injection sites for NSP cells could also form tumors as well, although the observation period had to be increased to eight weeks. There was no significant difference in tumorigenicity *in vivo* between SP and NSP cells, which suggested that although *CSC* was enriched in SP cells, it may contain both SP and NSP cells.

Indefinite self-renewal is one of the essential properties of stem cells. A stem cell could undergo asymmetric division continuously, producing one cell that retains self-renewal ability and differentiates into a mature cell. Stem cells and progenitor cells have the property of

anchorage-independent growth[22]. Therefore, soft agar and sphere formation assays can be used to test the self-renewal ability of stem cells *in vitro*[23,24]. In serial soft agar and sphere formation assays, single cells isolated from primary colonies coming from SP cells could form a secondary colony, suggesting that SP cells have the self-renewal capacity.

Bmi-1 is a transcriptional repressor belonging to the polycomb group of transcription factors. It has been shown to be important in the self-renewal of both normal and leukemic stem cells, as well as neuronal stem cells[25]. Oct-4 is a POU homeodomain transcription factor that is a key regulator of self-renewal in embryonic stem cells[26]. Cellular expression of Oct-4 is believed to have the capacity for self-renewal[27]. Moreover, the self-renewal ability of cancer stem cells could drive tumorigencity[28]. Oct-4 and Bmi-1 overexpression in SP cells isolated from KATO Ⅲ was observed, which might reflect the property of self-renewal of SP cells.

While ABCG2 can efflux the DNA binding dye Hoechst 33342, which could induce an SP phenotype, MDR1 is the best-studied member of the ABC transporter superfamily of genes[29]. The expression level of *ABCG2* and *MDR1* mRNA was higher in SP than in NSP cells as determined by RT-PCR analysis, which contributes to the chemotherapeutic resistance of SP cells, and thus may be a target for cancer therapy.

COMMENTS

Background

Gastric cancer is the second leading cause of cancer-related deaths worldwide. Cancer stem cells have the characteristics of longevity, self-renewal and proliferation. It is believed that cancer stem cells are responsible for the heterogeneity and relapse of certain cancers including gastric cancer.

Research frontiers

Side population (SP) cells can efflux the DNA-binding dye Hoechst33342 through an adenosine-triphosphate-binding cassette (ABC) membrane transporter and harbor stem cell-like characteristics. Recent studies suggested that SP cell analysis can be used to identify cancer stem cell populations. However, there has been no report identifying SP cells from the gastric cancer cell lines.

Innovations and breakthroughs

This is the first study to analyze SP and non-side population (NSP) cells in several human gastric cancer cell lines. The clonogenic ability of SP cells isolated from KATO Ⅲ was higher than that of NSP cells in soft agar and sphere forming assays. *Oct-4* and *Bmi-1* overexpression in SP cells isolated from KATO Ⅲ might reflect the property of self-renewal of SP cells. Moreover, *ABCG2* and *MDR1* overexpression contributes to the chemotherapeutic resistance of SP cells, and thus may be a target for cancer therapy.

Applications

SP cells could be used as a therapeutic target and for preventing relapse and drug-resistance in gastric cancer.

Terminology

Cancer stem cells are characterized by its self-renewal capacity, differentiation potential, and cancer-initiating ability. Side population cells expressing the ABC transporter were distinguished from whole cell population. Recent studies demonstrated that SP cells could be characterized as cancer stem cells in primary tissues and tumor cell lines, therefore, it was postulated to be responsible for tumor development and recurrence.

Peer review

Overall, this paper is very well written with good English and solid methodology. The biggest issue is that the content is not new. Basically, what the authors did was to confirm and characterize the side population cells in gastric cancer cell lines, which is not surprising. With that said, it is believed no one has done it in these cells, and it still may somewhat be informative to readers.

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BRIEF ARTICLE

7-difluoromethoxyl-5,4′-di-n-octylgenistein inhibits growth of gastric cancer cells through downregulating forkhead box M1

Hong-Lin Xiang, Fei Liu, Mei-Fang Quan, Jian-Guo Cao, Yuan Lv

Hong-Lin Xiang, Fei Liu, Mei-Fang Quan, Jian-Guo Cao, Yuan Lv, Laboratory of Medical Engineering, Medical College, Hunan Normal University, Changsha 410013, Hunan Province, China

Author contributions: Xiang HL, Liu F, Quan MF and Lv Y performed the majority of experiments; Cao JG designed the study and wrote the manuscript.

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Correspondence to: Jian-Guo Cao, Professor, Laboratory of Medical Engineering, Medical College, Hunan Normal University, Changsha 410013, Hunan Province, China. caojianguo2005@yahoo.com.cn
Telephone: +86-731-8912434 Fax: +86-731-8912417

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Abstract

AIM: To investigate whether the 7-difluoromethoxyl-5, 4′-di-n-octylgenistein (DFOG), a novel synthetic genistein analogue, affects the growth of gastric cancer cells and its mechanisms.

METHODS: A series of genistein analogues were prepared by difluoromethylation and alkylation, and human gastric cancer cell lines AGS and SGC-7901 cultured *in vitro* were treated with various concentrations of genistein and genistein analogues. The cell viability was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The cells were incubated by DFOG at different concentrations. The growth inhibitory effects were evaluated using MTT and clonogenic assay. The distribution of the phase in cell cycle was analyzed using flow cytometric analysis with propidium iodide staining. The expression of the transcription factor forkhead box M1 (FOXM1) was analyzed by reverse transcription-polymerase chain reaction and Western blotting. The expression levels of CDK1, Cdc25B, cyclin B and $p27^{KIP1}$ protein were detected using Western blotting.

RESULTS: Nine of the genistein analogues had more effective antitumor activity than genistein. Among the tested analogues, DFOG possessed the strongest activity against AGS and SGC-7901 cells *in vitro*. DFOG significantly inhibited the cell viability and colony formation of AGS and SGC-7901 cells. Moreover, DFOG efficaciously arrested the cell cycle in G2/M phase. DFOG decreased the expression of FOXM1 and its downstream genes, such as CDK1, Cdc25B, cyclin B, and increased $p27^{KIP1}$ at protein levels. Knockdown of FOXM1 by small interfering RNA before DFOG treatment resulted in enhanced cell growth inhibition in AGS cells. Up-regulation of FOXM1 by cDNA transfection attenuated DFOG-induced cell growth inhibition in AGS cells.

CONCLUSION: DFOG inhibits the growth of human gastric cancer cells by down-regulating the FOXM1 expression.

Key words: Gastric cancer; 7-difluoromethoxyl-5,4′-di-n-octylgenistein; Genistein; Forkhead box M1; Therapeutic action

Peer reviewers: Soon-Sun Hong, Professor, Inha University School of Medicine, Clinical Research Center, 2F, Jeongsuk Bld, 3-ga, Shinheung-dong, Incheon 400-712, South Korea; Hikaru Nagahara, Professor, Department of Gastroenterology, Aoyama Hospital, Tokyo Women's Medical University, 2-7-13 Kitaaoyama, Minato-ku, Tokyo 107-0061, Japan

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INTRODUCTION

Genistein, 5,7,4'-trihydroxylisoflavone, as one of the active constituents of soybean products, has been reported to possess anti-cancer activities[1,2]. But probably because of its low solubility in both water and organic solvent, genistein has a very low bioavailability. The introduction of the fluorine or fluoride-bearing alkyl such as CF_3 or HCF_2 into organic molecules dramatically changes their physiological, physical and chemical properties[3]. To our best knowledge, the introduction of fluorine moiety into the aryl part of the flavonoid molecule can enhance their biological activities, including anti-bacterial, anti-fungal and anti-viral activities[3]. Fluorinated 3, 4-dihydroxychalcones have illustrated interesting biological activities, including anti-peroxidation and antitumor activity *in vitro*[4]. We have previously reported that introduction of CF_3 or HCF_2 group into chrysin (5,7-dihydroxylflavone) molecule can improve their anticancer activities[5-7]. Recently, we synthesized a series of difluoromethoxylated genistein analogues and determined their protective effects against vascular endothelial cells[8]. However, there are few studies which reported the anti-cancer effect of fluorinated genistein analogues.

Gastric cancer is one of the most common malignancies in the world and its incidence and mortality rank first in China[9]. Recent data indicate that the mortality of gastric cancer in China tends to increase and it severely threatens the health and life of people[10]. At present, surgery and chemotherapy remain as the main modalities in the management of gastric cancer, but the curative effect of the existing chemotherapeutic drugs is not satisfactory, which cause numerous side effects. Therefore, it has been a focus to search for new drugs capable of preventing and treating gastric cancer and other malignancies. Gastric cancer has been shown to have activated forkhead box protein M1 (FOXM1) signaling pathway[11]. The FOXM1 belongs to a family of evolutionary conserved transcriptional regulators that were characterized by the presence of a DNA-binding domain called the forkhead box or winged helix domain[12]. It has been shown that FOXM1 signaling plays an important role in cellular developmental pathways, and activation of FOXM1 signaling is associated with carcinogenesis[13]. FOXM1 signaling is frequently up-regulated in cancers, including lung, breast, pancreatic and gastric cancer[11,14-16]. Moreover, FOXM1 has been shown to regulate transcription of cell cycle genes, including Cdc25B, CDK1, cyclin B and $p27^{KIP1}$[13,17]. Recently, it has been reported that FOXM1 expression could serve as an independent predictor of a poor survival of gastric cancer patients[11]. Studies by Wang *et al*[18] have shown that genistein may inhibit FOXM1 activation in pancreatic cancer cells, leading to apoptotic cell death. Therefore, it is believed that the targeted inactivation of FOXM1 could represent a promising strategy for the development of novel selective anti-cancer therapies.

In the present study, we investigated whether the growth inhibitory effects of genistein and the novel synthetic genistein analogue 7-difluoromethoxyl-5,4'-di-n-octylgenistein (DFOG) on gastric cancer cells could be attributed to modulation of FOXM1 activity. We found that DFOG and genistein down-regulated the FOXM1 expression and its downstream genes, including cdc25B, CDK1, cyclin B and up-regulated $p27^{KIP1}$, resulting in the growth inhibition of gastric cancer cells. These results provide strong evidences to support that FOXM1 is a rational target in gastric cancer, and the targeted inactivation of FOXM1, especially by genistein and its analogue DFOG, may provide new insight into the strategy development for better prevention of tumor progression and/or treatment of gastric cancer.

MATERIALS AND METHODS

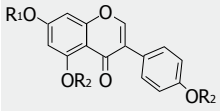
Cell culture and reagents

Human gastric cancer cell lines AGS and SGC-7901 were purchased from China Center for Type Culture Collection (CCTCC, Wuhan, China). Cells were maintained in RPMI 1640 medium (Invitrogen, Carlsbad, CA, United States) supplemented with 10% fetal bovine serum, 2 mmol/L glutamine, 100 mg/L penicillin, and 100 mg/L streptomycin, and incubated in a humidified atmosphere of 5% CO_2 at 37 ℃. The difluoromethoxylated genistein analogues 2,4a-4h were prepared by the method described elsewhere[8] (Table 1). Primary antibodies for FOXM1, CDK1, cyclin B, $p27^{KIP1}$ and Cdc25B were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, United States). Anti-β-actin antibody and Horseradish peroxidase-conjugated rabbit anti-mouse secondary antibody were purchased from Santa Cruz Biotechnology. Lipofectamine 2000 was purchased from Invitrogen. Protease inhibitor cocktail, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and all other chemicals were obtained from Sigma (St. Louis, MO, United States). Genistein (Sigma) and the difluoromethoxylated genistein analogues were dissolved in dimethyl sulfoxide (DMSO) to make a 10 mmol stock solution and were added directly to the media at different concentrations before use.

MTT assay

Cells were seeded in a 96-well plate at a density of 5000 cells/well as described previously[19]. After incubation for 24 h to allow cell attachment, different concentrations of genistein and genistein analogues (0.1, 0.3, 1.0, 3.0, 10.0 and 30.0 μmol/L) were added to each well and cultured for 24 h. Medium was removed and then incubated with 5.0 mg/mL MTT for 4 h. Then supernatant was removed after centrifugation. Finally, 100 μL of DMSO was added and absorbance at 570 nm wavelength (A_{570}) was measured by means of an Enzyme-labeling instrument (ELX-800 type; Bio-Tek, Shanghai, China). Relative cell proliferation inhibition rate = (1 - average A_{570} of the experimental group/average A_{570} of the control group) × 100%. The IC_{50} (defined as the drug concentration which 50% cell viability was inhibited) was as-

Table 1 Structures of genistein and its difluoromethylated derivatives



	Compound	R1	R2
1	Genistein (5,7,4′-trihydroxylisoflavone)	H	H
2	7-difluoromethyl genistein	CHF_2	H
4a	7-difluoromethyl-5,4′-dimethyl genistein	CHF_2	CH_3
4b	7-difluoromethyl-5,4′-diethyl genistein	CHF_2	CH_3CH_2
4c	7-difluoromethyl-5,4′-di-n-propyl genistein	CHF_2	n-C_3H_7
4d	7-difluoromethyl-5,4′-di-benzyl genistein	CHF_2	$C_6H_5CH_2$
4e	7-difluoromethyl-5,4′-diheptyl genistein	CHF_2	n-C_7H_{15}
4f	7-difluoromethyl-5,4′-di-n-octyl genistein	CHF_2	n-C_8H_{17}
4g	7-difluoromethyl-5,4′-didecyl genistein	CHF_2	n-$C_{10}H_{21}$
4h	7-difluoromethyl-5,4′-diisobutyl genistein	CHF_2	iso-C_4H_9

sessed from the dose-response curves using GraphPad Prism program (Version 4, GraphPad Software).

Clonogenic assay

Cells were plated in 24-well plates at a density of 300 cells/well for 24 h, prior to the addition of various concentrations of DFOG (1, 5 and 10 μmol/L) and 10 μmol/L genistein. After 48 h of treatment, the drug-containing medium was removed and replaced with complete growth medium. Medium was changed every 3 d for 8-10 d until visible colonies formed. Colonies were simultaneously fixed and stained with 0.5% crystal violet in methanol, and manually counted. Individually stained colonies in each well were counted and the colony formation fraction was calculated as follows: colony number/(number of cells seeded × plating efficiency), where plating efficiency is equivalent to the colony number divided by the number of cells seeded in the drug-free medium.

Cell cycle analysis by flow cytometry

Cells were plated in 6-well plates at a density of 1 000 000 cells/well for 24 h, prior to the addition of various concentrations (1, 5 and 10 μmol/L) of DFOG and 10 μmol/L genistein. After 24 h of treatment, cells were harvested, and DNA content was stained for 15 min at 37 ℃ with a solution containing 0.4% Triton X-100 (Sigma), 50 μg/mL of propidium iodide (Sigma), and 2 μg/mL of DNase-free RNase (Roche, United States). The cells were then analyzed for cell cycle perturbation using a FACSCalibur (FACS 420, Becton Dickinson, United States). The CellQuest program was used to quantitate the distribution of cells in each cell cycle phase: G1, S and G2/M.

Reverse transcription-polymerase chain reaction

Total RNA was extracted using Trizol reagent (Life Technologies, Gaithersburg, MD, United States). The integrity of the RNA was checked by 2% agarose gel electrophoresis. Approximately 2 μg RNA was reversely transcribed following the protocol of the Super Script™ first-strand synthesis system (Invitrogen Corporation, Carlsbad, CA, United States). The cDNAs encoding FoxM1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) genes were amplified by polymerase chain reaction (PCR) as follows: denaturation at 94 ℃ for 30 s, annealing at 63 ℃ for 30 s and elongation at 72 ℃ for 45 s. Primer sequence was designed using Primer. For FoxM1, the forward primer was 5'-AACCGCTACTTGACATTGG-3' and reverse primer 5'-GCAGTGGCTTCATCTTCC-3'. A housekeeping gene, GAPDH was used as the internal control. The forward primer was 5'-ACCCAGAAGACTGTGG ATGG-3', and the reverse primer was 5'-TGCTGTAGCCAAATTCGTTG-3'. PCR products were analyzed by agarose (2%) gel electrophoresis.

Plasmids and transfections

FOXM1 small interfering RNA (siRNA) and control siRNA were obtained from Santa Cruz Biotechnology. The FOXM1 cDNA plasmid was purchased from OriGene Technologies Inc (Rockvile, MD, United States). Human gastric cancer AGS cells were transfected with FOXM1 siRNA and cDNA, respectively, using Lipofectamine 2000 (Invitrogen) as described by Wang *et al*[20].

Western blotting analysis

Western blotting analysis was carried out as previously described[21]. Cells were lysed in lysis buffer by incubation for 20 min at 4 ℃. The protein concentration was determined using the Bio-Rad assay system (Bio-Rad, Hercules, CA, United States). Total proteins were fractionated using sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto Polyvinylidene fluoride membrane (Millipore, United States). Anti-FOXM1, anti-CDK1, anti-cyclin B, anti-$p27^{KIP1}$, anti-cdc25B and anti-β-actin rabbit polyclonal antibodies were used as primary antibodies. The signals were detected using an ECL Advance Western blotting analysis system (Amersham Pharmacia Biotech Inc., Piscataway, NJ, United States).

Statistical analysis

The SPSS 15.0 software package (SPSS Inc, Chicago, IL, United States) was used for the statistical analysis. Data were expressed as mean ± SD. The means of multiple groups were compared with one-way analysis of variance, after the equal check of variance, and the pairwise comparisons among the means were performed using the least significant difference method. Statistical comparison was also performed with two-tailed *t* test when appropriate. A $P < 0.05$ was considered as statistically significant.

RESULTS

Effects of genistein and genistein analogues on the cell viability of gastric cancer cells

First, we examined the effects of genistein and the genistein analogues on the viability of AGS and SGC-7901

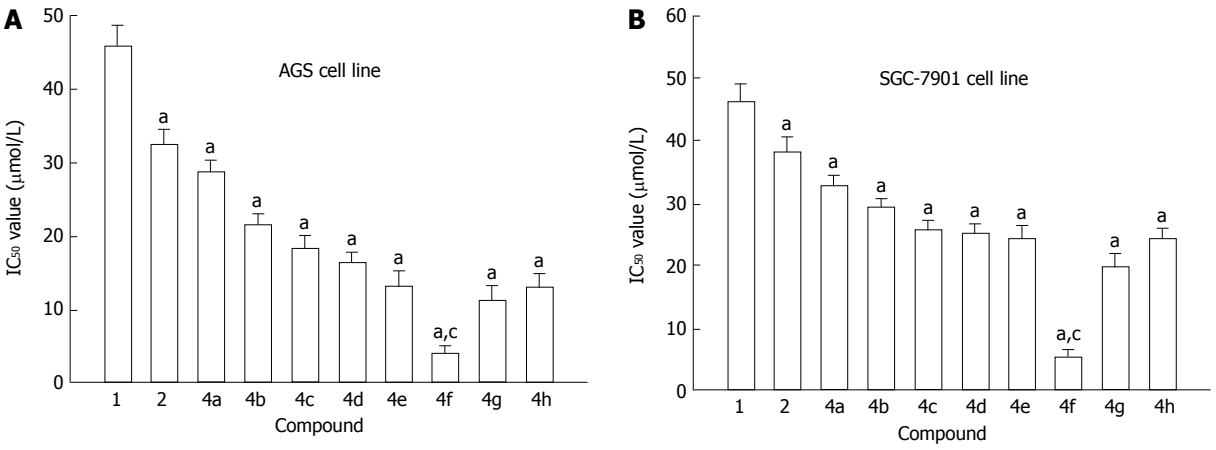


Figure 1 Inhibition of cell viability by genistein and genistein analogues. A: AGS cell line; B: SGC-7901 cell line. $^{a}P < 0.05$ *vs* treatment with genistein; $^{c}P < 0.05$ *vs* treatment with genistein or other genistein analogues. 1: Genistein (5,7,4'-trihydroxylisoflavone); 2: 7-difluoromethyl genistein; 4a: 7-difluoromethyl-5,4'-dimethyl genistein; 4b: 7-difluoromethyl-5,4'-diethyl genistein; 4c: 7-difluoromethyl-5,4'-di-n-propyl genistein; 4d: 7-difluoromethyl-5,4'-di-benzyl genistein; 4e: 7-difluoromethyl-5,4'-diheptyl genistein; 4f: 7-difluoromethyl-5,4'-di-n-octyl genistein; 4g: 7-difluoromethyl-5,4'-didecyl genistein; 4h: 7-difluoromethyl-5,4'-diisobutyl genistein.

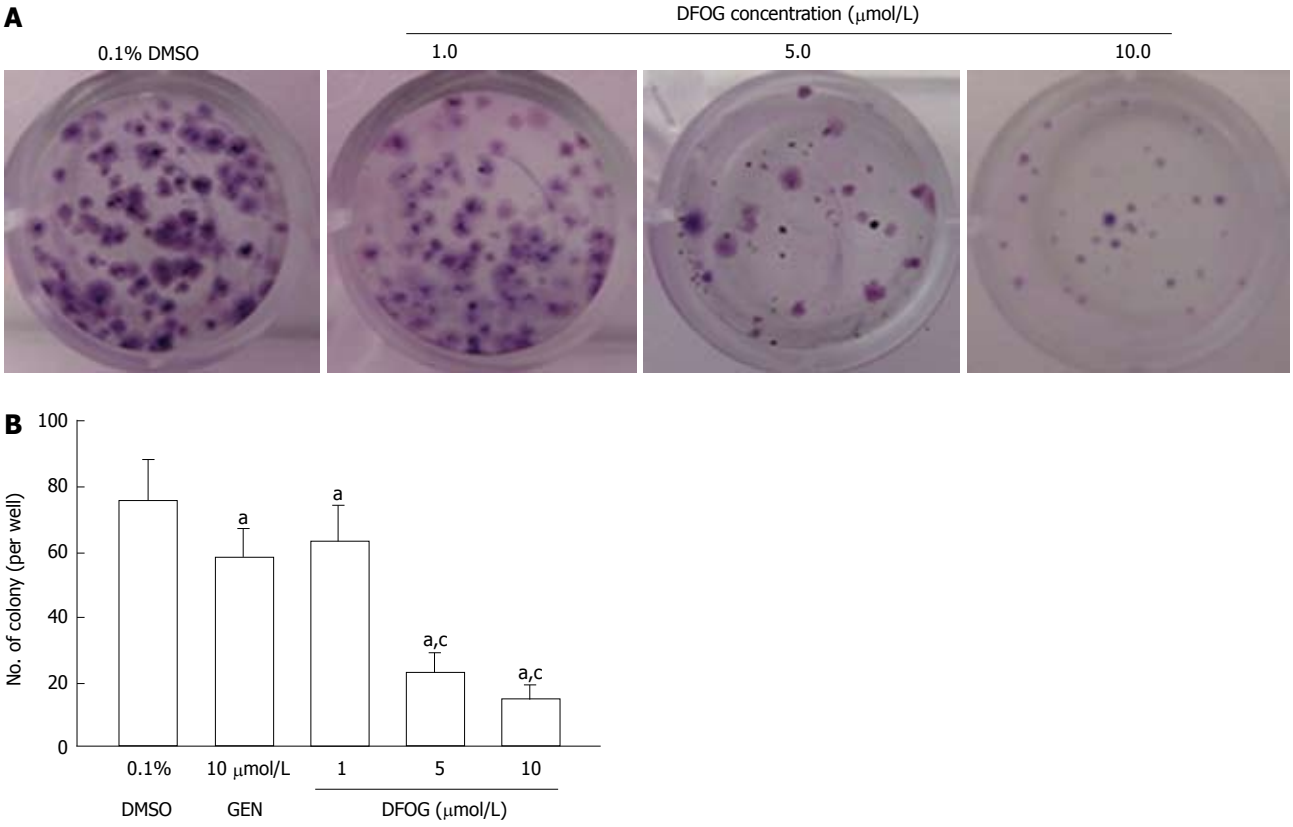


Figure 2 Decrease of colony number and inhibition of colony formation by 7-difluoromethoxyl-5,4'-di-n-octylgenistein. A: Decrease of colony number by 7-difluoromethoxyl-5,4'-di-n-octylgenistein (DFOG); B: Inhibition of colony formation by DFOG and genistein in AGS cell line. $^{a}P < 0.05$ *vs* treatment with dimethyl sulfoxide (DMSO); $^{c}P < 0.05$ *vs* treatment with 10 μmol/L genistein (GEN) or 1 μmol/L DFOG.

cells using MTT assay. Nine of difluoromethylated genistein analogues had higher effective antitumor activities than genistein. Among the aforementioned analogues, DFOG showed the strongest activity against AGS and SGC-7901 *in vitro* (Figure 1A and B). IC_{50} of DFOG was 3.9 μmol/L for AGS cells and 5.2 μmol/L for SGC-7901 cells, the potency of DFOG was 11.7 and 8.9 as much as that of the lead compound, genistein (IC_{50} was 45.9 μmol/L for AGS cells and 46.3 μmol/L for SGC-7901 cells).

Effects of DFOG on the colony formation of gastric cancer cells

Next, we tested the effects of DFOG on cell growth by clonogenic assay. DFOG treatment resulted in a significant inhibition of colony formation of AGS cells

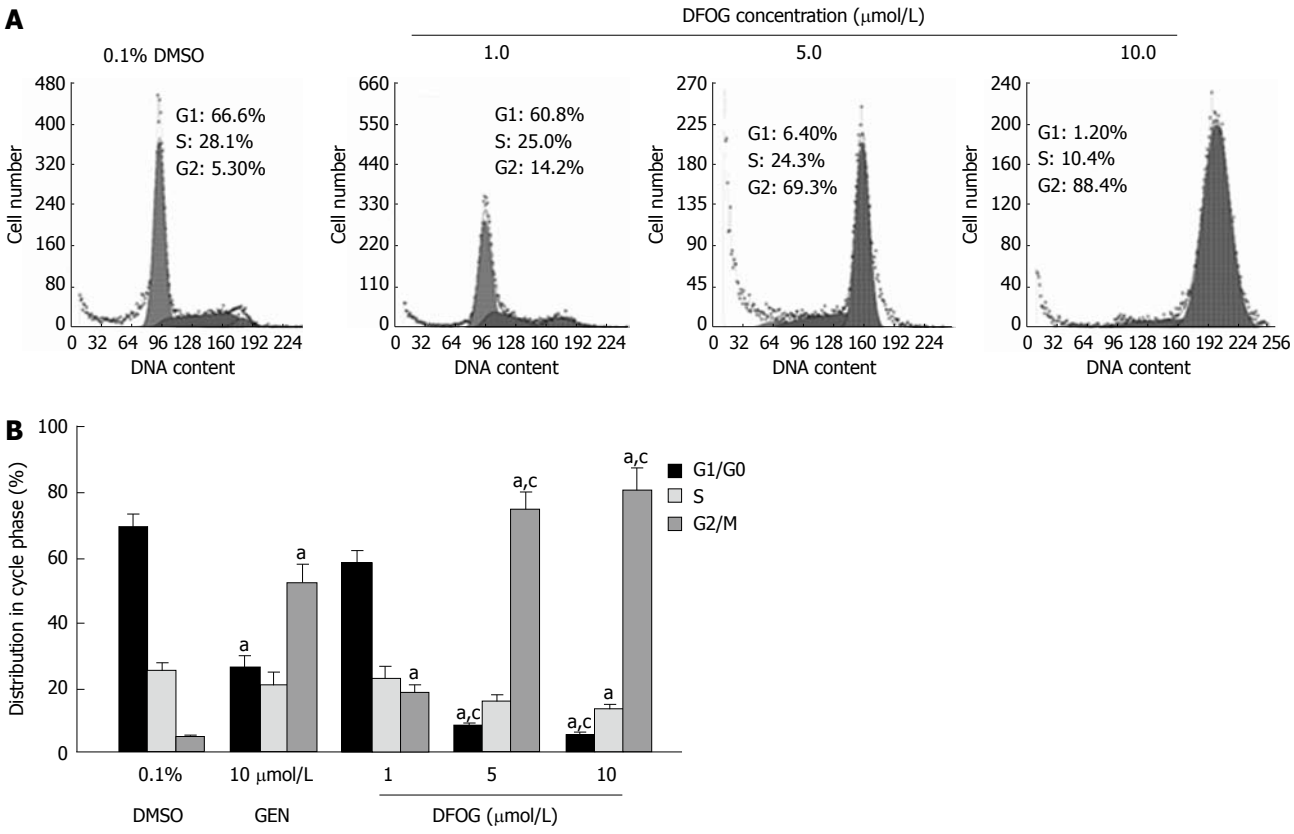


Figure 3 Increase of cells in G2/M phase and induction of cell cycle arrest in G2/M phase by 7-difluoromethoxyl-5,4'-di-n-octylgenistein. A: Increase of cells in G2/M phase by 7-difluoromethoxyl-5,4'-di-n-octylgenistein (DFOG); B: Induction of cell cycle arrest in G2/M phase by DFOG and genistein in AGS cell line. $^{a}P < 0.05$ *vs* treatment with dimethyl sulfoxide (DMSO); $^{c}P < 0.05$ *vs* treatment with 10 μmol/L genistein (GEN) or 1 μmol/L DFOG.

compared with controls (Figure 2A and B). Similar results were observed in SGC-7901 cells (data not shown). These data suggests that DFOG inhibits the growth of gastric cancer cells.

Effects of DFOG on the distribution of cell cycle phase in gastric cancer cells

To assess whether the loss of cell survival could in part be attributed to the induction of cell cycle arrest, we evaluated the effects of DFOG treatment on the distribution in cell phase using flow cytometry with propidium iodide staining. As shown in Figure 3A and B, in gastric cancer cell line AGS, DFOG treatment caused a significant accumulation of cells in the G2/M phase and a marked decrease in the G1/G0 phase compared with control cells. Similar results were observed in SGC-7901 cells (data not shown). These results provided convincing data that DFOG could induce the growth inhibition and arrest of cell cycle in G2/M phase in gastric cancer cells.

Effects of DFOG on FOXM1 expression in gastric cancer cells

The studies by Wang *et al*[18] have shown that FOXM1 signaling is over-expressed in pancreatic cancer and is involved in promotion of cell growth, and thus considered as a putative target for drug development. Therefore, we investigated whether DFOG could regulate FOXM1 signaling pathway. FOXM1 mRNA and protein expression in AGS cell line treated with DFOG and genistein for 24 h were decreased in a concentration-dependent manner (Figure 4A and B). We also found that FOXM1 protein expression was down-regulated by DFOG and genistein in SGC-7901 cells (Figure 4C).

Effects of DFOG on the expression of FOXM1 downstream target genes in gastric cancer cells

It is well known that FOXM1 has several downstream target genes, such as CDK1, Cdc25B, cyclin B, and $p27^{KIP1}$. We used Western blotting analysis to determine the expression of these genes, and found that DFOG and genistein inhibited the expression of CDK1, Cdc25B, cyclin B, and increased $p27^{KIP1}$ at the protein levels in AGS and SGC-701 cells (Figure 5A and B).

Effects of down-regulation of FOXM1 expression by siRNA on DFOG-induced cell growth inhibition in AGS cells

Down-regulation of FOXM1 by siRNA transfection showed less expression of FOXM1 protein in AGS cells, as confirmed by Western blot (Figure 6A).The down-regulation of FOXM1 expression significantly inhibited cell viability induced by DFOG (Figure 6B). DFOG plus FOXM1 siRNA inhibited cell growth to a greater degree compared with DFOG alone. FOXM1 siRNA transfection induces arrest of cell cycle in G2/M phase in AGS cells (Figure 6C). These results provide some molecular evidences suggesting that the DFOG-induced inhibition

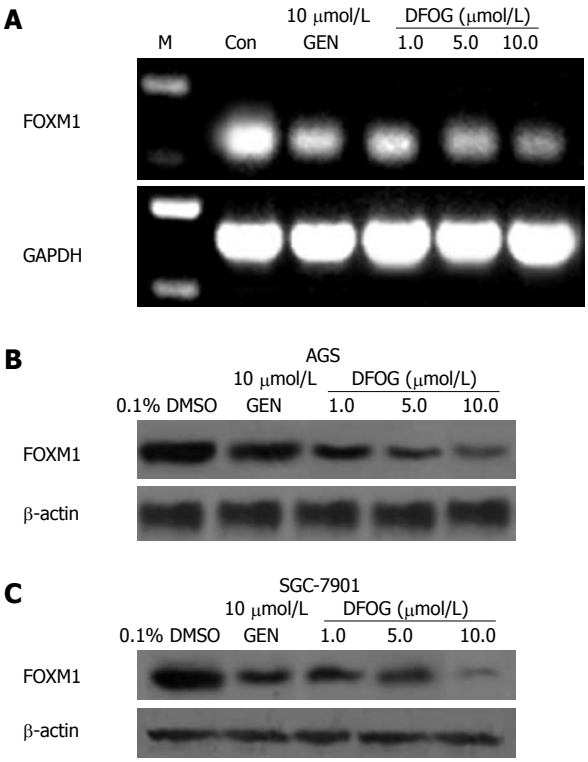


Figure 4 Down-regulation of forkhead box M1 expression by 7-difluoromethoxyl-5,4'-di-n-octylgenistein and genistein in AGS and SGC-7901. A: mRNA level using reverse transcription-polymerase chain reaction in AGS cell line; B: Protein level using Western blotting in AGS cell line; C: Decrease of forkhead box M1 (FOXM1) protein expression in SGC-7901 cell line. DMSO: Dimethyl sulfoxide; GEN: Genistein; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

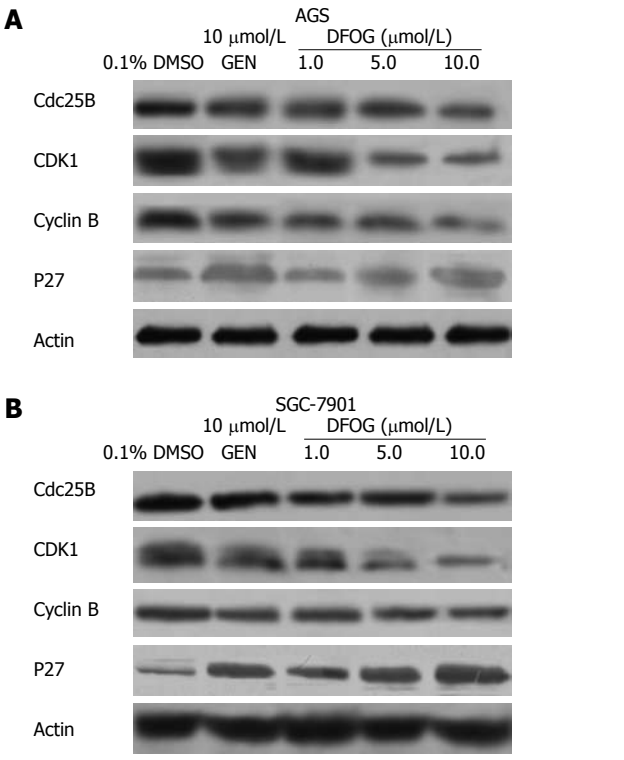


Figure 5 Modulation of the protein expressions of forkhead box M1 downstream target genes by 7-difluoromethoxyl-5,4'-di-n-octylgenistein and genistein. A: AGS cell line; B: SGC-7901 cell line. DMSO: Dimethyl sulfoxide; GEN: Genistein; DFOG: 7-difluoromethoxyl-5,4'-di-n-octylgenistein.

of the growth is mediated *via* inactivation of FOXM1 in gastric cancer cells.

Effects of over-expression of FOXM1 by cDNA transfection on DFOG-induced cell growth inhibition in AGS cells

Up-regulation of FOXM1 by cDNA transfection showed over-expression of FOXM1 protein in AGS cells, as confirmed by Western blotting (Figure 7A). The over-expression of FOXM1 rescued DFOG-induced cell viability inhibition to a certain degree (Figure 7B). These findings suggested that DFOG-inhibited cell growth is in part attributed to inactivation of FOXM1 signaling pathway in gastric cancer cells.

DISCUSSION

FOXM1 signaling has been demonstrated to maintain a balance between cell proliferation, differentiation and apoptosis, suggesting that abnormal activation of *FOXM1* gene is one of the characteristics of human cancers[22]. Increasing studies have shown over-expression of *FOXM1* gene in human cancer cells and tissues[14,23,24]. Thus, the development of agents targeting FOXM1 is likely to have a significant therapeutic impact on the treatment of human cancers, including gastric cancer. FOXM1 could be down-regulated by some drugs, such as antibiotic thiazole compound Siomycin A, thiostrepton, and epidermal growth factor receptor inhibitor gefitinib[25-27]. These observations clearly suggest that chemical compounds that target FOXM1 may act as anti-cancer drugs[27]. Wang *et al*[18] have shown that genistein may inhibit FOXM1 activation in pancreatic cancer cells, leading to apoptotic cell death. In the present study, we used two human gastric cancer cell lines, AGS and SGC-7901, which have high expression of FOXM1, and found that DFOG, a novel synthetic genistein analogue, and genistein could induce significant growth inhibition in the two cell lines, as evidenced by both MTT and clonogenic assay. Furthermore, DFOG and genistein inhibited the expression of FOXM1 and its target genes. Therefore, DFOG and genistein could mediate the cell growth inhibition partly *via* inactivation of FOXM1. Down-regulation of FOXM1 by siRNA together with DFOG treatment inhibited cell growth to a greater degree in AGS cells as compared with DFOG treatment alone. Up-regulation of FOXM1 by cDNA transfection showed over-expression of FoxM1 protein as confirmed by Western blotting analysis, and this over-expression in FOXM1 attenuated DFOG-induced cell growth inhibition in AGS cells. In view of these findings, we strongly believe that inactivation of FOXM1 by DFOG and genistein results in the down-regulation of its target genes, which are mechanistically linked with DFOG and genistein induced cell growth inhibition.

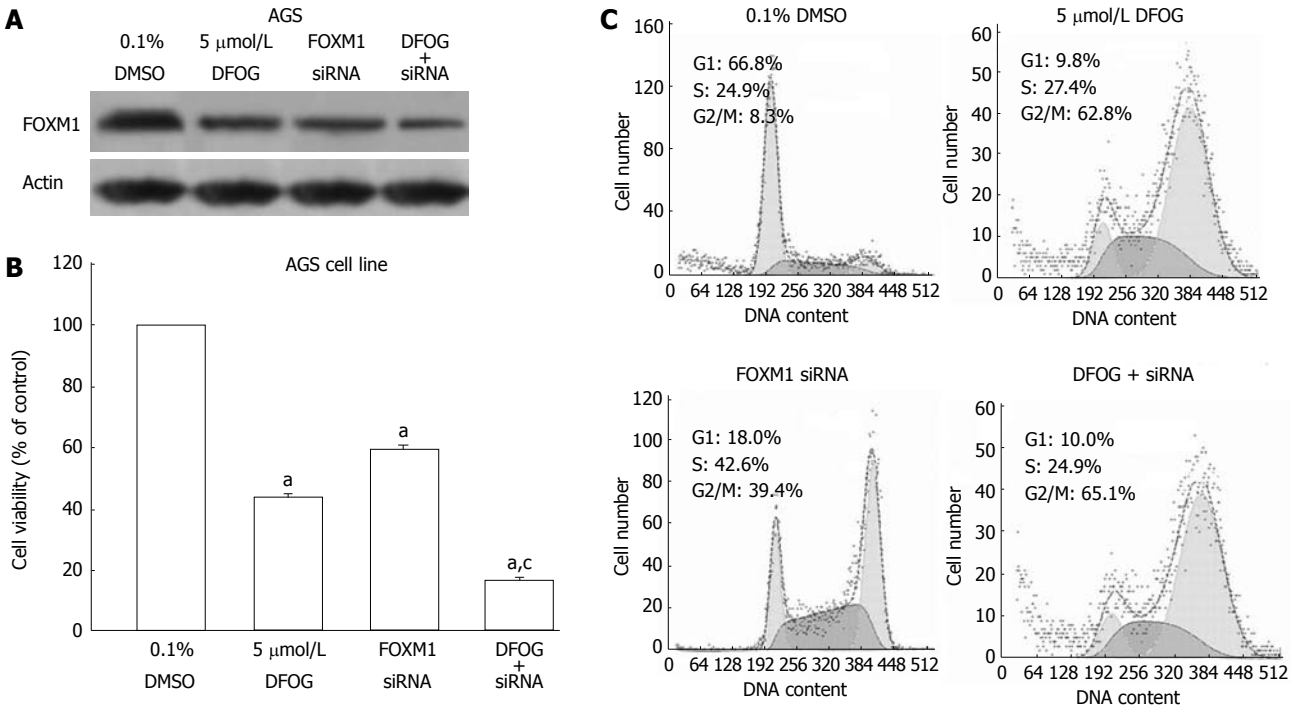


Figure 6 Forkhead box M1 small interfering RNA enhances the down-regulation of forkhead box M1 protein expression by 7-difluoromethoxyl-5,4'-di-n-octylgenistein and effects of forkhead box M1 small interfering RNA transfection or 7-difluoromethoxyl-5,4'-di-n-octylgenistein in AGS cell line. A: Western blotting analysis; B: The cell viability inhibitory effects of 7-difluoro methoxyl-5,4'-di-n-octylgenistein (DFOG) by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylte trazolium bromide assay; C: Both on cell cycle distribution in AGS cells. $^{a}P < 0.05$ *vs* treatment with dimethyl sulfoxide (DMSO); $^{c}P < 0.05$ *vs* treatment with 5 μmol/L DFOG or forkhead box M1 (FOXM1) small interfering RNA (siRNA) alone.

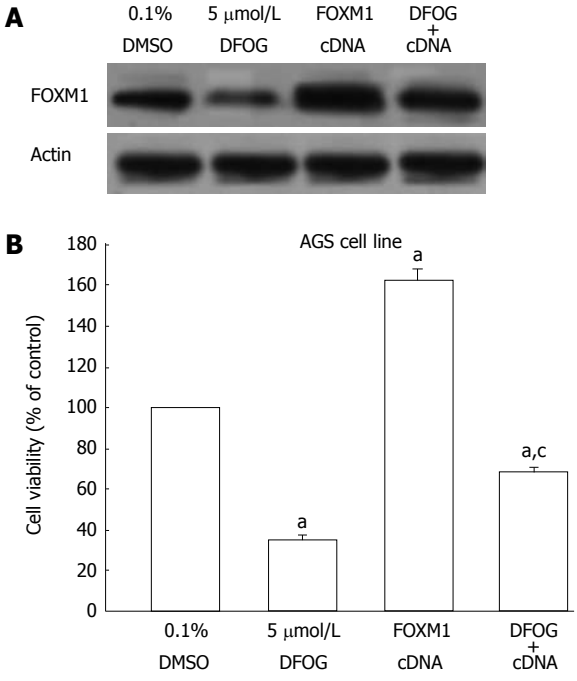


Figure 7 Forkhead box M1 cDNA transfection reduces the down-regulation of forkhead box M1 protein expression by 7-difluoromethoxyl-5,4'-di-n-octylgenistein in AGS cell line. A: Western blotting analysis; B: The cell viability inhibitory effects of 7-difluoro methoxyl-5,4'-di-n-octylgenistein (DFOG) by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylte trazolium bromide assay. $^{a}P < 0.05$ *vs* treatment with dimethyl sulfoxide (DMSO); $^{c}P < 0.05$ *vs* treatment with 5 μmol/L DFOG or forkhead box M1 (FOXM1) cDNA transfection alone.

Genistein has been found to induce G2 arrest and inhibit proliferation in a variety of cancer cell lines[28]. Because down-regulation of FOXM1 by DFOG reduced cell growth, we postulated that cell growth inhibition might result from cell cycle arrest in any specific phase of the cell cycle. We did find that FOXM1 down-regulation increased cell population in the G2/M phase and decreased cells in the G1/G0 and S phase. We also observed a marked reduction in cyclin B, Cdc25B and CDK1 expression in DFOG-treated cells. It is known that Cdc25B, cyclin B and CDK1 are the major effectors of the G2/M checkpoint response[29]. Diminished G1-S progression and growth rate was associated with increased expression of the *Cdk*I protein $p21^{CIP}$ and $p27^{KIP1}$, which have negative effects on cell cycle machinery by binding to various cyclin-Cdk complexes and inhibiting their activities[24,30]. In our study, the decreased cyclin B, cdc25B and Cdk1 and the increased expression of CdkI proteins, $p27^{KIP1}$, were strongly correlated with the altered cell cycle distribution phenotype and growth suppression. These results suggest that FOXM1 affects the gastric cancer cell cycle by regulating the expression levels of some cyclins (cyclin B), CDK (CDK1), CDK modulator (cdc25B) and CDK inhibitors ($p27^{KIP1}$).

In summary, we presented experimental evidence which strongly supports the role of DFOG, a novel synthetic genistein analogue, as an anti-tumor agent mediated through inactivation of FOXM1 signaling pathway.

However, further in-depth studies are needed to identify how DFOG could regulate the FOXM1 pathway, and to assess the anti-tumor activity mediated by the inactivation of FOXM1 either by genistein and DFOG or other synthetic compounds in pre-clinical animal models for the successful treatment of gastric cancer in the future. It is also tempting to speculate that the inactivation of FOXM1 together with the treatment of gastric tumor cells with conventional agents could be a useful strategy toward better treatment of human malignancies, especially gastric cancer.

COMMENTS

Background

Gastric cancer is one of the most common malignancies in the world and its incidence and mortality rank first in China. Recent data indicate that the mortality of gastric cancer in China tends to increase and it severely threatens the health and life of people. New therapeutic agents for this malignant disease are urgently needed.

Research frontiers

Genistein, 5,7,4'-trihydroxylisoflavone, a major component of soybean products, has been reported to possess anticancer activities. The authors synthesized a series of difluoromethoxylated genistein analogues and determined their protective effects against vascular endothelial cells. There have been few studies focusing on the anticancer effect of fluorinated genistein analogues.

Innovations and breakthroughs

The authors investigated whether the inhibitory effects of genistein and the novel synthetic genistein analogue 7-difluoromethoxyl-5, 4'-di-n-octylgenistein (DFOG) on the growth of gastric cancer cells could be attributed to modulation of Forkhead Box M1 (FOXM1) activity. It was found that DFOG and genistein down-regulated the FOXM1 expression and its downstream genes, including Cdc25B, CDK1, cyclin B and up-regulated $p27^{KIP1}$, resulting in the inhibition of gastric cancer cell growth.

Applications

These results provide strong evidences for the first time to support that FOXM1 is a rational target in gastric cancer, and the targeted inactivation of FOXM1, especially by genistein and its analogue DFOG, and provided new insight into strategies for better prevention of tumor progression and/or treatment of gastric cancer.

Terminology

Genistein, 5,7,4'-trihydroxylisoflavone, as one of the active constituents of soybean products, has been reported to possess anti-cancer activities. The DFOG is a novel synthetic genistein analogue.

Peer review

The novel anti-cancer drugs are mandatory to overcome cancer cells resistant to conventional anti-cancer drugs. In this paper the authors identified DFOG to be a good anti-cancer drug in gastric cancer cells. In addition, they also obtained the results to show FOXM1 as one of target molecules of DFOG. All data presented in this paper is acceptable.

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CASE REPORT

A case report of abdominal distention caused by herpes zoster

Su-Rong Zhou, Chuan-Yu Liu

Su-Rong Zhou, Department of Dermatology, The Affiliated Yantai Yuhuangding Hospital of Qingdao University Medical College, Yantai 264000, Shandong Province, China
Chuan-Yu Liu, Department of Neurology, The Affiliated Yantai Yuhuangding Hospital of Qingdao University Medical College, Yantai 264000, Shandong Province, China
Author contributions: Liu CY managed the case; Zhou SR wrote the paper.
Correspondence to: Chuan-Yu Liu, PhD, Department of Neurology, the Affiliated Yantai Yuhuangding Hospital of Qingdao University Medical College, No. 20 Yudong Road, Yantai 264000, Shandong Province, China. lcy32@126.com
Telephone: +86-535-6691999 Fax: +86-535-6240341
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Abstract

Gastrointestinal complications caused by herpes zoster are extremely rare. Here, we described a case of abdominal distention caused by herpes zoster. The patient was a 59-year-old female who suffered from unexplained paroxysmal and a burning pain on the right part of her waist and abdomen, accompanied by abdominal distention. Intestinal pseudo-obstruction was diagnosed by abdominal radiography. Distention of the right abdominal wall was still apparent after one month. In this report, we found that recovery from abdominal distention caused by herpes zoster is difficult and may require surgical intervention.

Key words: Abdominal distention; Herpes zoster; Intestinal pseudo-obstruction; Ogilvie's syndrome

Peer reviewer: Dr. Shailendra Kapoor, University of Illinois, 7487 Sherwood Crossing Place No. 302, Mechanicsville, VA 23111, United States

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INTRODUCTION

Herpes zoster is a viral disease that typically manifests as a dermatological condition, marked by an irritating skin rash with blisters that is often limited to one side of the body; although rare, it has also been implicated in unusual gastrointestinal complications[1]. We treated a patient with abdominal distention caused by herpes zoster and report it below.

CASE REPORT

A 59-year-old female patient suffered from unexplained paroxysmal and a burning pain on the right part of her waist and abdomen for 2 wk. An erythra appeared on the affected area one week ago and the pain was exacerbated, interrupting the patient's sleep. A diagnosis of herpes zoster was made and the patient was administrated with valaciclovir (0.3 g *bid*), vitamin B_{12} (0.5 mg im *qd*), and ibuprofen and codeine phosphate sustained tablets (50 mg *bid*) for 3 wk. The patient had abdominal distention accompanied by dyspepsia, abdominal bloating and constipation, which showed no apparent changes after motilium treatment. Upon physical examination, laminal erythema was found on her left waist and abdomen accompanied by papulovesicles and small vesicles. The erythra showed zonal distribution not exceeding the midline. The abdomen was asymmetric with left distention, a lax abdominal wall and hyperalgesia. Two air-fluid levels appeared on the abdominal radiograph, which was diagnosed as intestinal pseudo-obstruction by surgical consultation. After treatment with fasting, fluid infusion,

enema and moxifloxacin (1 tablet *qd* × 3), the symptoms were relieved after 5 d. The diagnosis was modified as intestinal pseudo-obstruction caused by herpes zoster. Continued treatment included mecobalamin (1 tablet *tid*) to nourish the nerve and for heat-clearing and a detoxifying soft capsule (2 pills *tid*) to clear away heat and toxic materials and relax the bowels. After 15 d, the red spots disappeared, and the vesicles dried up, changed to scabs and dropped off, leaving anomalous pigmentation of the skin. At the same time, the pain was apparently alleviated, but the distention of the right abdominal wall and constipation persisted. On follow-up one month later, the distention of the right abdominal wall was still apparent, but the partial sense of pain decreased and the constipation was relieved.

DISCUSSION

Herpes zoster is a herpetic skin disease caused by varicella-zoster virus (VZV) infection, which can infect the surrounding sensory and motor nerves and their dominated skin areas. When the abdomen is involved, the infection can cause paralysis of the abdominal wall muscle[2], which may involve the autonomic nerve, the myenteron and small intestine smooth muscles, leading to the manifestation of intestinal pseudo-obstruction (Ogilvie's syndrome)[1]. The present patient was an overweight, middle-aged female. Due to two pregnancies, her abdomen became flabby. VZV infection in this area may cause nerve paralysis, a decrease in abdominal muscle tonus and abdominal distention. Simultaneously, indigestion, constipation and intestinal pseudo-obstruction appear due to the involvement of the autonomic nerves. Further consultation after one month showed no improvement of the abdominal wall distention. As recovery from this type of nerve paralysis is difficult, surgical intervention is sometimes necessary.

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Dr. Leif Percival Andersen, MD, Department of Infection Control 9101, Copenhagen University Hospital, Rigshospitalet, Juliane Maries Vej 18, DK-2100 Copenhagen, Denmark

Ekaterini Chatzaki, PhD, Assistant Professor, Laboratory of Pharmacology, Faculty of Medicine, Dragana, Alexandroupolis, 68100 Thrace, Greece

Edward J Ciaccio, PhD, Research Scientist, Department of Medicine, HP 804, Columbia University, 180 Fort Washington Avenue, New York, NY 10032, United States

Andrew S Day, MB, ChB, MD, FRACP, AGAF, Associate Professor, Department of Paediatrics, University of Otago, Christchurch, P.O. Box 4345, Christchurch 8140, New Zealand

Giuliana Decorti, MD, PhD, Department of Life Sciences, University of Trieste, Via L. Giorgieri n° 7, 34127 Trieste, Italy

Magdy El-Salhy, MD, PhD, Professor, Chief Gastroenterologist, Section for Gastroenterology, Department of Medicine, Stord Helse-Fonna Hospital, Box 4000, 54 09 Stord, Norway

Francesco Franceschi, MD, PhD, Assistant Professor, Internal Medicine, Catholic University of Rome, Gemelli Hospital, Largo A. Gemelli, 8, 00168 Rome, Italy

Oliver Grundmann, PhD, Clinical Assistant Professor, Department of Medicinal Chemistry, College of Pharmacy, University of Florida, 1600 SW Archer RD, Room P6-20, Gainesville, FL 32610-0484, United States

Masahiro Iizuka, MD, PhD, Director, Akita Health Care Center, Akita Red Cross Hospital, 3-4-23, Nakadori, Akita 010-0001, Japan

Eric R Kallwitz, MD, Assistant Professor, Department of Medicine, University of Illinois, 840 S Wood Street, 7th Floor, MC 787, Chicago, IL 60612, United States

Hyo-Cheol Kim, MD, Clinical Assistant Professor in Vascular Intervention Section, Department of Radiology, Seoul National University Hospital, 28 Yongon-dong, Chongno-gu, Seoul 110-744, South Korea

Young Chul Kim, PhD, Professor of Toxicology, Seoul National University, College of Pharmacy, San 56-1 Shinrim-Dong, Kwanak-Ku, Seoul 151-742, South Korea

Javier San Martín, Chief, Gastroenterology and Endoscopy, Sanatorio Cantegril, Av. Roosevelt y P 13, 20100 Punta del Este, Uruguay

B Mittal, PhD, Professor, Department of Genetics, Sanjay Gandhi Medical Institute, Lucknow 226014, India

Hikaru Nagahara, MD, PhD, Professor, Aoyama Hospital, Tokyo Women's Medical University, 2-7-13 Kita-Aoyama, Minatoku, Tokyo 107-0061, Japan

Atsushi Nakajima, Professor, Division of Gastroenterology, Yokohama City University Graduate School of Medicine, 3-9 Fuku-ura, Kanazawa-ku, Yokohama 236-0004, Japan

Noriko Nakajima, MD, PhD, Associate Professor, Division of Gastroenterology and Hepatology, Department of Internal Medicine, Nihon University School of Medicine, 1-8-13 Kandasurugadai Chiyoda-ku, Tokyo 101-8309, Japan

Samiran Nundy, MD, Department of Surgical Gastroenterology and Liver Transplantation, Sir Ganga Ram Hospital, New Delhi 110060, India

Bronislaw L Slomiany, PhD, Professor, Research Center, C-875, UMDNJ-NJ Dental School, 110 Bergen Street, PO Box 1709, Newark, NJ 07103-2400, United States

Branko Stefanovic, PhD, Department of Biomedical Sciences, College of Medicine, Florida State University, 1115 W.Call st, Tallahassee, FL 32306-4300, United States

Hans L Tillmann, Professor, Medizinische Klinik und Poliklinik II, University Leipzig, Philipp Rosenthal, Str. 27, 04103 Leipzig, Germany

Kentaro Yoshika, Associate Professor, Division of Gastroenterology, Department of I, Fujita Health University School of Medicine, 1-98 Dengakugakubo, Kutsukade, Toyoake 470-1190, Japan

Katsutoshi Yoshizato, PhD, Academic Advisor, Phoenix Bio Co., Ltd., 3-4-1 Kagamiyama, Higashihiroshima 739-0046, Japan

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American Gastroenterological Association Clinical Congress of Gastroenterology and Hepatology
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London, United Kingdom

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4th United Kingdom Swallowing Research Group Conference
London, United Kingdom

February 23, 2012
Management of Barretts Oesophagus: Everything you need to know
Cambridge, United Kingdom

February 24-27, 2012
Canadian Digestive Diseases Week 2012
Montreal, Canada

March 1-3, 2012
International Conference on Nutrition and Growth 2012
Paris, France

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Society of American Gastrointestinal and Endoscopic Surgeons Annual Meeting
San Diego, CA 92121, United States

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World Congress on Gastroenterology and Urology
Omaha, NE 68197, United States

March 17-20, 2012
Mayo Clinic Gastroenterology and Hepatology
Orlando, FL 32808, United States

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26th Annual New Treatments in Chronic Liver Disease
San Diego, CA 92121, United States

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San Antonio, TX 78249, United States

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Milwaukee, WI 53202, United States

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Singapore, Singapore

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New Orleans, LA 70166, United States

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Diffuse Small Bowel and Liver Diseases
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EUROSON 2012 EFSUMB Annual Meeting
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PancreasFest 2012
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2012 Joint International Neurogastroenterology and Motility Meeting
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3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

Organization as author

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5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

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6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

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15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http://www.cdc.gov/ncidod/eid/index.htm

Patent (list all authors)

16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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