ISSN 1007-9327 (print)
ISSN 2219-2840 (online)

World Journal of *Gastroenterology*

World J Gastroenterol 2016 June 7; 22(21): 4963-5136

Published by Baishideng Publishing Group Inc

World Journal of Gastroenterology

A peer-reviewed, online, open-access journal of gastroenterology and hepatology

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2014-2017

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World Journal of Gastroenterology

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Weekly Volume 22 Number 21 June 7, 2016

CASE REPORT

World Journal of Gastroenterology
Volume 22 Number 21 June 7, 2016

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NAME OF JOURNAL
World Journal of Gastroenterology

ISSN
ISSN 1007-9327 (print)
ISSN 2219-2840 (online)

LAUNCH DATE
October 1, 1995

FREQUENCY
Weekly

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PUBLISHER
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PUBLICATION DATE
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DOI: 10.3748/wjg.v22.i21.4963

World J Gastroenterol 2016 June 7; 22(21): 4963-4965
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

EDITORIAL

Surgical dilemmas in the management of colorectal liver metastases: The role of timing

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Conflict-of-interest statement: The authors have no conflict of interests.

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Received: February 23, 2016
Peer-review started: February 24, 2016
First decision: March 31, 2016
Revised: April 19, 2016
Accepted: May 4, 2016
Article in press: May 4, 2016
Published online: June 7, 2016

Abstract

Colorectal cancer (CRC) is an emerging health problem in the Western World both for its raising tendency as well as for its metastatic potential. Almost half of the patients with CRC will develop liver metastases during the course of their disease. The liver surgeon dealing with colorectal liver metastases faces several surgical dilemmas especially in the setting of the timing of operation. Synchronous resectable metastases should be treated prior or after induction chemotherapy? Furthermore in the case of synchronous colorectal liver metastases which organ should we first deal with, the liver or the colon? All these questions are set in the editorial and impulse for further investigation is put focusing on multidisciplinary approach and individualization of treatment modalities.

Key words: Colorectal cancer; Chemotherapy; Timing of surgery; Colorectal liver metastases; Liver first procedure; Multidisciplinary approach; Individualized treatment strategies

Core tip: The treatment of colorectal cancer with colorectal liver metastases is a challenge for the multidisciplinary medical team dealing with this problem. The timing of surgery both for synchronous as well as for metachronous metastases is always a matter of debate. Multidisciplinary approach and individualization of treatment strategies is suggested.

Dimitroulis D, Kouraklis G. Surgical dilemmas in the management of colorectal liver metastases: The role of timing. *World J Gastroenterol* 2016; 22(21): 4963-4965 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i21/4963.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i21.4963

Colorectal cancer (CRC) remains an important public health issue as it is the third leading cause of death for both men and women in the United States and the most frequent cause of cancer among patients aged 75 years and older[1]. Furthermore approximately 10%-25% of patients with CRC present at the time

of diagnosis with liver metastases (CRLM) while 25% will develop liver metastases in the future, a fact that means almost half of the patients with CRC will develop liver metastatic disease. While in the past the presence of multiple or enlarged CRLM was a sign towards palliative treatment the progress in liver surgery, medical oncology and interventional radiology has allowed us to perform liver resections for colorectal liver metastases with intention to treat[1].

The surgeon who confronts with colorectal liver metastases faces several problems. Should I operate first or is it better for the patient to receive neoadjuvant treatment. And when it comes to the operation should I operate both liver and colon or separately and if separately which organ first. The conventional way of thinking in patients with resectable synchronous colorectal liver metastases is to offer an upfront operation and the reason for this attitude is the fear that CRLM will not respond to chemotherapy and that during the time of chemotherapy the liver tumors will grow and become unresectable so that the patient will lose the possibility of a curative liver operation[2]. Though, in this case scenario there is always a possibility to develop post-operative complications that will delay the chemotherapeutical approach and the patient will not benefit from medical oncology. Furthermore if we choose chemotherapy prior to surgical resection the tumor load within the liver is assumed to decrease and therefore we could achieve a higher percentage of complete resection rate (R0 resection) as well as the ability to perform minor hepatectomies, another argument counting for neoadjuvant chemotherapy. In some cases we might as well have a complete response, a phenomenon called "vanishing metastases" in the pertinent literature, "where the dream of the oncologist becomes the nightmare of the surgeon", because even if we have a complete radiological response there are still some active tumor cells that require surgical resection. In order to avoid such a problem there several solutions proposed, like marking the metastatic lesions prior to oncological referral. However, the choice of chemotherapy prior to surgical resection will have favorable results only under the circumstance that the tumor is sensitive to the therapeutical regimen we choose, so a complete examination of the k-ras and b-raf should be performed. Another pitfall of this approach is the development of chemotherapy adverse effects that will delay surgery, especially if we take into consideration that almost all oncological agents develop liver toxicity (*e.g.,* blue liver)[3].

The second problem for the surgeon is whether to proceed to a combined liver and colon operation or a staged one. This problem has created a debate in the pertinent literature. The combined operation can theoretically solve the patient's surgical problem with one shot. This approach has a better impact on the patient's psychology as well as the financial aspect because we have one admission and one surgical procedure and in total decreased time of hospitalization. On the other hand a combined procedure can lead to an increased risk of adverse effects of both colectomy and hepatectomy and in that case the patient will face a delayed post-operative course and might lose the time window for chemotherapy. Most authors conclude that it would be better to avoid low anterior colectomies with major liver resections. For the rest of the cases there is no consensus and the decision should be individualized taking into consideration the tumor load, the patient's performance status and the experience of the institution[4]. Furthermore for the case the surgeon decides to proceed to a combined operation our group has published experimental data demostrating that if we prefer to start with the liver and perform an intermittent Pringle maneuver the post-operative outcomes are favorable[5].

There is also a great amount of patients who present with unresectable liver metastases or with metastatic disease to other organs apart from the liver (lungs, peritoneal deposits *etc.*) at initial diagnosis. Patients with colorectal liver metastases initially considered as unresectable should be under close follow up by the surgical team during chemotherapy. Classical therapeutical agents in combination with biological agents (monoclonal antibodies) have increased the resectability rate of these patients. Furthermore, the use of special maneuvers such as portal vein ligation or embolization together with the above mentioned chemotherapeutical agents enable the decrease of tumor load with a concomitant increase of remnant liver volume so that the number of patients who are candidates for liver resection raises even more. For patients who develop metastatic disease to other organs beside the liver the site of metastasis affects both the treatment and prognosis. In patients with colorectal metastases to liver and lung and in some cases also the peritoneum the most important factor that affects the prognosis is the resectability of the metastatic lesion or lesions, especially the liver metastases[6].

The management of a patient with synchronous colorectal liver metastases is a difficult, complicated and provocative problem to solve. With the evolution of science and technology the resectability rate has raised and more patients have favorable outcome. Surgeons are always enthusiastic but a lot of factors must be taken into consideration before choosing the best approach for each patient. The decision for the optimal treatment should be individualized, based on the results of a tumor board taking into consideration the opinion of surgeons, radiologists and oncologists. However, the most important predictive factors are the patient's performance status, the tumor load and the biological behavior of the disease.

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P- Reviewer: Guo JC, Wang ZX **S- Editor**: Ma YJ
L- Editor: A **E- Editor**: Ma S

World Journal of Gastroenterology

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DOI: 10.3748/wjg.v22.i21.4966

World J Gastroenterol 2016 June 7; 22(21): 4966-4976
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

TOPIC HIGHLIGHT

2016 Liver Transplantation: Global view

How important is donor age in liver transplantation?

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Author contributions: Lué A, Solanas E, Baptista P and Serrano MT wrote the paper; Lué A, Solanas E, Lorente S, Araiz JJ, García-Gil A and Serrano MT contributed to data collection; Solanas E, Lorente S, Baptista P and Serrano MT revised and contributed to paper structure.

Conflict-of-interest statement: The authors have no conflict of interest to report.

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Received: March 20, 2016
Peer-review started: March 22, 2016
First decision: March 31, 2016
Revised: April 15, 2016
Accepted: May 4, 2016
Article in press: May 4, 2016
Published online: June 7, 2016

Abstract

The age of liver donors has been increasing in the past several years because of a donor shortage. In the United States, 33% of donors are age 50 years or older, as are more than 50% in some European countries. The impact of donor age on liver transplantation (LT) has been analyzed in several studies with contradictory conclusions. Nevertheless, recent analyses of the largest databases demonstrate that having an older donor is a risk factor for graft failure. Donor age is included as a risk factor in the more relevant graft survival scores, such as the Donor Risk Index, donor age and Model for End-stage Liver Disease, Survival Outcomes Following Liver Transplantation, and the Balance of Risk. The use of old donors is related to an increased rate of biliary complications and hepatitis C virus-related graft failure. Although liver function does not seem to be significantly affected by age, the incidence of several liver diseases increases with age, and the capacity of the liver to manage or overcome liver diseases or external injuries decreases. In this paper, the importance of age in LT outcomes, the role of donor age as a risk factor, and the influence of aging on liver regeneration are reviewed.

Key words: Liver transplantation; Liver regeneration; Graft survival; Old donor; Aging

Core tip: Because of a donor shortage, the use of grafts from old donors has become widespread. Donor age is

related to worse outcomes after liver transplantation, higher rates of graft failure, biliary complications and a worse graft survival. In recipients with hepatitis C, the impact of donor age is even more evident. Aging-related changes at the hepatocellular level may contribute to a decreased capacity of the liver to manage or overcome liver diseases and injuries. This review summarizes the evidence regarding the impact of donor age on liver transplantation outcomes.

Lué A, Solanas E, Baptista P, Lorente S, Araiz JJ, Garcia-Gil A, Serrano MT. How important is donor age in liver transplantation? *World J Gastroenterol* 2016; 22(21): 4966-4976 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i21/4966.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i21.4966

INTRODUCTION

In the recent years a considerable change in donor age distribution of liver transplantation (LT) has been observed, as shown in the figures from the European Liver Transplant Registry (ELTR), with a rising percentage of livers proceeding from donors older than 60 years. In 1989, only 1% of livers proceeded from donors over 60 years of age. This rate escalates to 15% in 1999, 20% in 2001, and 29% in 2009[1,2]. In Spain, between 1984 and 1995, only 11.5% of donors were age 55 years or older, while between 2011 and 2012, 61.8% of donors were 55 years or older (Figure 1)[3]. The United Network for Organ Sharing (UNOS), in the United States, reports that in 1989, 2.4% of donors were age 50 years or older, but this rate increased to 29% in 1999 and to 33% in 2013 (Figure 2).

The impact of donor age on LT has been evaluated in different studies with contradictory results. Many studies did not observe differences in graft survival according to donor age[4,5], on the contrary others report an increases of complication rates and poorer survival following transplantation from older donors[6,7]. Furthermore, a relationship has been described between allografts obtained from older donors and a faster progression of fibrosis after LT in recipients infected with hepatitis C virus (HCV)[8,9].

IMPACT OF DONOR AGE ON LIVER TRANSPLANT OUTCOMES

Deceased donor liver transplant

Studies based on institutional registries have evaluated the effects of donor age on patient and graft survival in the largest patient series[1-3,6,7]. In the ELTR, the 1-year survival of patients who received transplants between 1998 and 2001 was similar for all donor age groups[2]. In a recent analysis of the same ELTR database, graft survival was significantly higher if the organs proceed

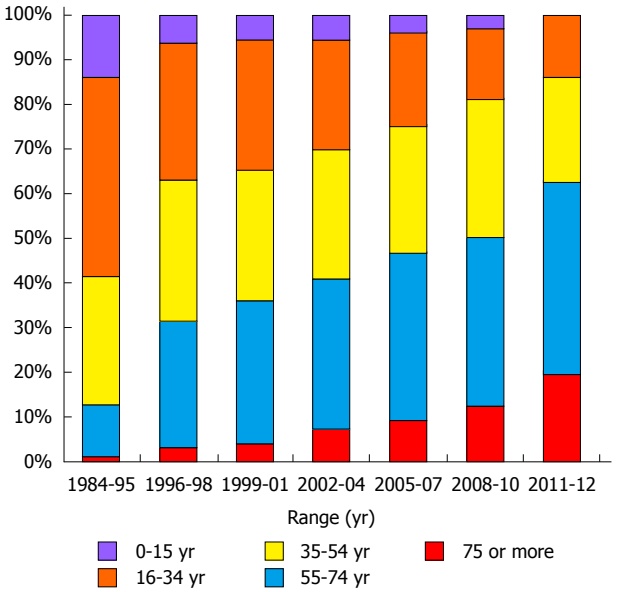

Figure 1 Change in distribution of donor age in recent years. Source: Spanish Liver Transplant Registry.

from donors younger than 55 years *vs* donors older than 65 years (65% *vs* 57%, $P < 0.0001$)[1]. An analysis of the data collected by the Spanish Registry for Liver Transplantation between 1991 and 2013 shows that donor age influences LT outcome (Figure 3). LT performed with deceased donors over age 55 years had a slight but significant worsening in actuarial graft survival one year after LT compared with those realized with graft from donors younger than 55 years. The difference in graft survival between the two groups was more evident at 5 years after LT[3]. Feng *et al*[10] recently analyzed donor risk factors in LTs finding that donor age over 60 years was the strongest risk factor for graft failure. In this analysis of the data collected from the Scientific Registry of Transplant Recipients, donor age over 40 years and especially over 60 years, donation after cardiac death, and split/partial grafts were strongly associated with graft failure. In a retrospective analysis performed using data obtained from the UNOS, Reese *et al*[11] found that performing LTs with donors who were ⩾ 45 years old increased the risk of graft failure at 90 d after transplantation. Moreover, these authors found that a combination of prolonged cold ischemia time and older donor age were associated with a decrease in graft survival after LT. We performed a prospective analysis to establish if donor age over 60 years could be a risk factor for higher incidence of complications or graft failure[12]. We did not observe differences in the initial graft function between groups. Moreover in the older donor group we did not observe any case of primary non-function and patient survival was not affected. Nevertheless, graft survival at 12 mo was decreased by about 15% in the older donor group, although patient survival was not affected.

Other studies show different results. Anderson *et*

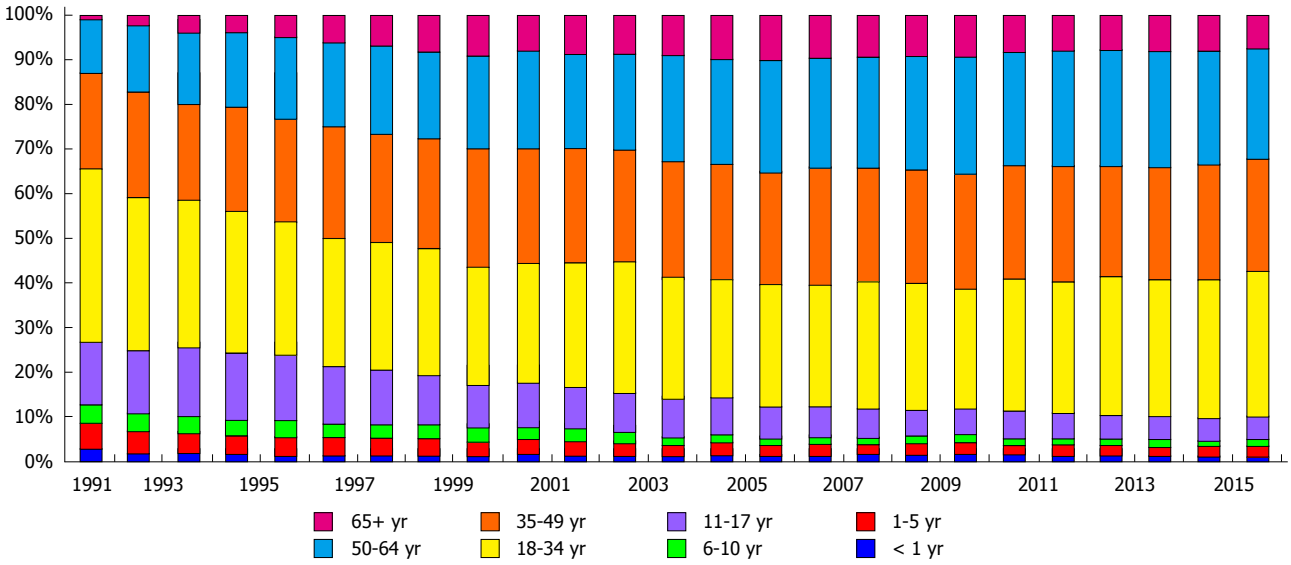

Figure 2 Change in distribution of donor age in recent years. Source: United Network for Organ Sharing reports.

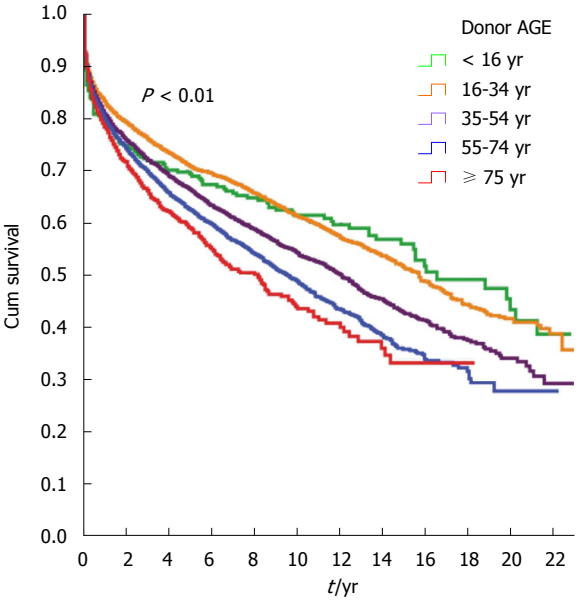

Figure 3 Graft survival depending on donor age. Source: Spanish Liver Transplant Registry.

al[13] analyzed 741 LTs performed between 1990 and 2007 and did not found significant difference in overall graft and patient survival with donors younger than 60 years compared to those aged 60 or older. However, when cases with donors ⩾ 60 years were compared with each other from different time period, the authors observed that the LT performed after 2001 had a better patient and graft survival. LT performed before 2001 had significantly longer cold ischemic times compared with those performed after 2001. From this study, these authors concluded that donor age *per se* is not a disadvantage for graft or patient survival, but that there was a possible interaction between donor age and other factors such as ischemia time.

Alamo *et al*[14] conducted a case-control single-center study and examined the outcomes of 129 livers transplanted from donors older than age 70 years. The authors observed no differences in survival but did identify a greater incidence of ascites and primary dysfunction, probably secondary to a delayed start in graft function. They recognized that recipient Model for End-Stage Liver Disease (MELD) score and cold ischemia time were parameters associated with a poor prognosis. In addition the authors found that some donor factors were associated with a poor prognosis: diabetes, hypertension, and weight greater than 90 kg. With these results, this group concluded that LT with liver grafts from elderly donors is safe but that the selection of donors and recipients must be done with care. Kim *et al*[15] retrospectively analyzed outcomes of LT using livers from donors age 65 years and older and tried to identify those factors that affected graft survival. The results indicated that these factors were hepatitis C as the etiology of liver disease, MELD score higher than 20, donor serum glucose level higher than 200 mg/dL at the time of liver recovery, and skin incision to aortic cross-clamp time longer than 40 min in the donor surgery. In the analysis, the authors observed that the 5-year cumulative graft survival rate of none, one, two, three, and four unfavorable characteristics was 100%, 82%, 81.7%, 39.3%, and 25%, respectively ($P < 0.05$). The authors suggested that the grafts from older donors should not be considerate useless based only on age and that in selected cases, they can result in good graft survival. All these studies are summarized in the Table 1.

Living donor liver transplant

Han *et al*[16] recently demonstrated that living donor LT (LDLT) using elderly donors, defined as those ⩾ 55 years of age, could be related with more serious complications and higher mortality rates. In that retrospective analysis including 604 LDLTs, the

Table 1 Studies that analyze impact of donor age on liver transplant outcomes

Ref.	Type of donor	Cut-off age	No. of patients	Outcomes
Adam *et al*[1]	Deceased donor	< 55 yr *vs* > 65 yr	80347	Higher graft survival with donors younger than 55 yr
Adam *et al*[2]	Deceased donor	Multiple age groups	41522	No differences in one-year survival
Cuervas-Mons *et al*[3]	Deceased donor	55 yr	18568	Lower graft 5-yr survival rate with older donors
Feng *et al*[10]	Deceased donor	60 yr	20023	Higher rate of graft failure with older donors
Reese *et al*[11]	Deceased donor	45 yr	14756	Higher rate of graft failure at 90 d after LT with older donors
Serrano *et al*[12]	Deceased donor	60 yr	149	Lower graft survival rate with older donors
Anderson *et al*[13]	Deceased donor	60 yr	741	No differences were observed
Alamo *et al*[14]	Deceased donor	70 yr	129	No differences were observed in selected recipients (non HCV, low MELD, younger than 60 yr)
Kim *et al*[15]	Deceased donor	65 yr	100	Donor age should not be an absolute contraindication
Han *et al*[16]	Living donor	55 yr	604	Higher mortality rate with older donors
Dayangac *et al*[17]	Living donor	50 yr	150	Higher rate of major complication with older donors
Ikegami *et al*[18]	Living donor	< 30 yr *vs* > 50 yr	34	Better graft function and regeneration rates with donors < 30 yr
Ikegami *et al*[19]	Living donor	50 yr	232	Higher rate of small for size syndrome with older donors
Iwamoto *et al*[20]	Living donor	50 yr	232	Worse survival and high bilirubin levels with older donors
Ono *et al*[21]	Living donor	< 30 yr *vs* > 50 yr	15	Lower regeneration rate a week after LT with older donors
Uchiyama *et al*[22]	Living donor	48 yr	321	Higher rate of small for size syndrome with older donors
Li *et al*[23]	Living donor	70 yr	129	No differences in recipient survival rate at 1, 3 and 5 yr
Wang *et al*[24]	Living donor	50 yr	159	No differences in recipient survival rate at 1, 3 and 5 yr

LT: Liver transplantations; HCV: Hepatitis C virus; MELD: Model for End-stage Liver Disease.

mortality rate was significantly higher in the elderly *vs* the younger donor group. The 5-year survival rate was 44.6% in the elderly group and 80.7% in the younger group, and the median overall survival was significantly shorter in the elderly group (31.2 ± 31.3 mo *vs* 51.4 ± 40.8 mo, $P = 0.014$). Biliary (41.7%) and arterial complications (16.7%) were the more frequent causes of death in the elderly group, which were both significantly higher than in the younger group. This study was limited because of its retrospective analysis that included a small number of patients in the elderly group; nevertheless, the results suggest that donor age directly affects overall survival and complication rate in LDLT.

Another recent study[17] demonstrated a significant association between surgical technique aspects and the rate of major complications when grafts from donors aged ⩾ 50 years are used. In LDLT, enlarging the limits of surgery is associated with more complications in elderly donors. With donors who are ⩾ 50 years old, these authors recommend avoiding right hepatectomy with middle hepatic vein harvesting or resulting in an estimated remnant liver volume less than 35%. Other reports suggest that donor age might have a major effect on recipient outcome in adult LDLT. Ikegami *et al*[18] demonstrated that LT performed with living donors ⩽ 30 years old resulted in better function and regeneration rates within the first month than those performed with donors > 50 years of age. However, the outcome was not affected by the age of the liver graft. In a further study[19], the same authors demonstrated a greater incidence of small-for-size syndrome in recipients from living donors older than 50 years compared to those transplanted with livers from donors ⩽ 50 years old. In addition, Iwamoto *et al*[20] reported significantly higher bilirubin levels and worse survival following transplantations using donors age 50 years or older. Recently, Ono *et al*[21] analyzed hepatic regeneration in living donors and observed that the regeneration rate a week after hepatectomy was significantly higher in donors who were ⩽ 30 years old than in those ⩾ 50 years old; however, the differences disappeared within a month after LT.

These results are consistent with the more recent work of Uchiyama *et al*[22], who retrospectively analyzed 321 consecutive LDLTs performed between 2004 and 2014 and found that donor age was a significant risk factor for small-for-size graft syndrome. In the conclusions, the authors suggest that the use of hepatic grafts from older donors should be avoided if possible to minimize post-transplant complications[22].

On the contrary Li *et al*[23], in a retrospective analysis, found no differences in complication rates and recipient survival at 1, 3, and 5 years. These data suggest that LDLT using older donors had no negative influence on the outcomes of both donors and recipients.

These results are consistent with other recent studies. Wang *et al*[24] analyzed the outcome of 159 LDLTs divided by donor age into older or younger than 50 years and found no significant difference in graft or recipient survival at 1, 3, and 5 years. However, the volume of red blood cells transfused during the surgical procedure was greater in the older donor group (1.900 mL *vs* 1.200 mL, $P = 0.023$). From these results, the authors suggested that LDLT with donors older than 50 years old is safe and that there are not significant adverse effects in terms of graft function and long-term donor and patient survival. All these studies are

Table 2 Variables included in the most relevant survival scores

Model	Variables included	Ref.
DRI	D-age, donor height, DCD, split, race, COD, allocation, CIT	Feng *et al*[10]
ET-DRI	D-age, DCD, Partial/Split, GGT, allocation, rescue allocation	Braat *et al*[25]
SOFT	D-age, COD, donor creatinine, R-age, R-BMI, previous OLT, previous abdominal surgery, R-albumin, dialysis, UNOS status, MELD score, encephalopathy, PVT, ascites, portal bleed, life support, allocation, CIT	Rana *et al*[26]
D-MELD	D-age, MELD score	Halldorson *et al*[27]
BAR	MELD score, CIT, R-age, D-age, previous OLT, life support	Dutkowski *et al*[28]

COD: Cause of death; CIT: Cold ischemia time; DCD: Donation after cardiac death; DRI: Donor risk index; D-age: Donor age; ET-DRI: Eurotrasplant donor risk index; SOFT: Survival outcomes following liver transplantation; R-age: Recipient age; OLT: Orthotopic liver transplant; PVT: Portal vein thrombosis; D-MELD: Donor age Model for End-stage Liver Disease; BAR: Balance of risk.

summarized in the Table 1.

DONOR AGE AS RISK FACTOR IN PROGNOSTIC SCORES

In the past several years, donor quality has been decreasing. Some studies have tried to detect the most important risk factors and to develop several mathematical formulas designed to predict graft outcome. All of them include donor age as a risk factor (Table 2). Feng *et al*[10] performed one of the most relevant studies; this group used the UNOS database to identify eight donor factors predicting graft failure after transplantation (donor age, donor height, donation after cardiac death, split liver donor, black race, vascular accident as cause of death, regional sharing, and cold ischemia time). A donor risk index (DRI) was developed, using these risk factors, to predict the isolated and cumulative effects of these variables on graft survival. Recipients of grafts with a DRI < 1.2 had a graft survival higher than 80% per year *vs* 71.4% in those transplanted with organs with a DRI > 2. In that study, donor age over 60 years was the strongest risk factor for graft failure (relative risk = 1.53 with a donor > 60; 1.65 if > 70). However, this index is not easily applicable in every country. In Europe, Eurotrasplant region database analysis showed that donor age ($P < 0.0001$), donation after cardiac death ($P = 0.001$), split/partial liver ($P < 0.0001$), latest serum GGT gamma-glutamyl transpeptidase ($P = 0.006$), allocation ($P < 0.0001$), and rescue allocation ($P = 0.005$) were significantly associated with an increased risk of graft failure. These six factors were used to construct a "new theoretical Eurotransplant risk index"[25].

Because post-transplantation patient survival depends on both the preoperative medical condition and donor quality, physicians often face the difficult decision of whether to accept high-risk donor liver offers for high-risk patients. Thus, in contrast with DRI, the Survival Outcomes Following Liver Transplantation (SOFT) score includes donor and recipient factors and also ischemia times[26]. The overall result of the score could guide the clinician to either accept or reject the offered allograft, based on the projected risk calculation. Authors proposed that cold ischemia time might be estimated when the offer is performed. Donor age > 70 is the donor variable that has a greater weight in the SOFT score[26]. Halldorson *et al*[27] tried to identify poor donor/recipient matches that could help to direct allocation of organs to recipients in which the survival is greatest, maximizing the benefit of donor livers. They created the D-MELD score, which was calculated as the product of the MELD score and donor age and was demonstrated to be highly predictive of post-LT survival. A D-MELD cut-off of 1600 identified donor/recipient combinations with significantly poorer survival. This score could predict excessive donor/recipient match risk and improve resource use. Another risk score described by Dutkowski *et al*[28] is the balance of risk system, which detects unfavorable combinations of donor and recipient factors. It analyzes six factors including donor age. In summary, donor age is a variable included in all main scores that analyze the risk of death and graft loss after LT and is one of the factors that weighs the most in these models.

LIVER AGE AS RISK FACTOR IN LIVER TRANSPLANT COMPLICATIONS

Donor age also has been described as a risk factor in development of some specific complications such as biliary and aggressive recurrence of HCV. Here we describe the studies that support these data.

Biliary complications

In recent years, numerous studies have shown that donor age may be related to a higher prevalence of biliary strictures. Thorsen *et al*[29] found that LTs performed with donors older than 75 years presented more biliary complications when compared with those patients who received a graft from donors aged 20 to 49 years (29.6% *vs* 13%). However, survival did not differ between groups. Verdonk *et al*[30] found that the incidence of anastomotic strictures (AS) increased from 5.3% before 1995 to 16.7% after 1995, possibly related to an increase in the use of grafts from donors with extended criteria. Similarly, Sundaram *et al*[31] found that biliary AS rate increases after the introduction of MELD for graft allocation (6.4% in the pre-MELD era *vs* 15.4% in the post-MELD era). Transplantation in the post-MELD era was an independent risk factor for biliary AS (OR = 2.30;

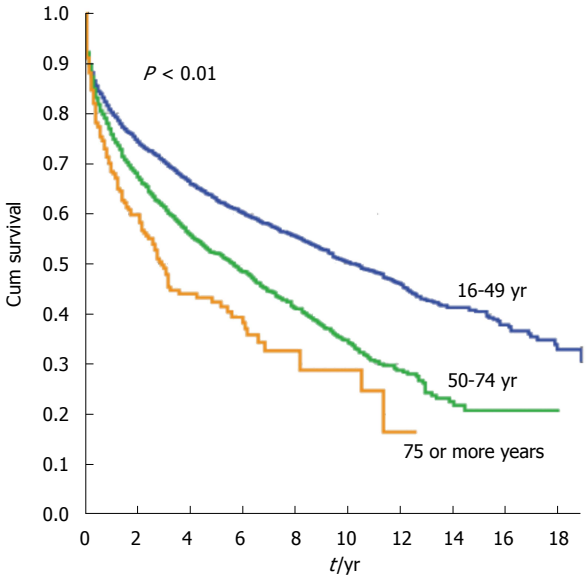

Figure 4 Graft survival in hepatitis C virus-infected patients depending on donor age. Source: Spanish Liver Transplant Registry.

95%CI: 1.60-3.32, $P = 0.001$). Other risk factors were donor age (OR = 1.01; 95%CI: 1.00-1.02, $P = 0.015$), a prior bile leak (OR = 2.24; 95%CI: 1.32-3.76, $P = 0.003$), and a choledochocholedochostomy (OR = 2.22; 95%CI: 1.23-4.06, $P = 0.008$). Nevertheless, in most studies, age was not a risk factor for AS, but it is in non-AS. Lüthold *et al*[32] in a recent study in a pediatric population showed that risk factors for intrahepatic biliary strictures were donor age over 48 years (increase 1.09 fold) and MELD score higher than 30 (increase 1.2 fold). Heidenhain *et al*[33] analyzed nearly 2000 patients retrospectively and found that donor age ($P = 0.028$) and cold ischemia time ($P = 0.002$) were significant risk factors for the development of ischemic-type biliary lesions after liver transplant.

In the study performed by our group and mentioned above, we detected that non-anastomotic biliary strictures (NAS) were four times more frequent in the older donor group. In multivariate analysis (stepwise multiple logistic regression was performed) receiving a graft from a donor 60 years or older (OR = 4.2; 95%CI: 1.24-13.35, $P < 0.01$) and arterial complications (AC) (OR = 67; 95%CI: 11.39-394, $P < 0.0001$) were both independent risk factors associated with NAS. Almost one half of the LT patients with NAS did not have arterial thrombosis. In the logistic regression analysis donor age ⩾ 60 years, emerge as an independent risk factor for intrahepatic non-ischemic strictures (OR = 15.4; 95%CI: 1.42-168.1, $P = 0.024$)[12]. NAS development in these cases could be related to ischemia-reperfusion injury. Despite there were no differences in ischemia time between the two groups it is possible that grafts from older donors were less tolerant to ischemic reperfusion injury. Similar complications have been described with non-beating-heart liver donors; the incidences of both NAS and ischemia-reperfusion injury is higher than with beating-heart donors[34]. Experimental data demonstrated that ischemia-reperfusion injury significantly affects the biliary tree. *In vitro* studies performed on human samples have demonstrated histological and molecular changes in the bile duct that are related to ischemic injury and indicate that biliary tract is the most sensitive structure to this type of injury[35]. Cells from bile duct are more exposed to re-oxygenation damage because they express lower levels of glutathione than hepatocytes[36].

In a recent work, Ghinolfi *et al*[37] demonstrated than LT with liver of donors older than 80 years of age is associated with a higher rate of NAS. Nevertheless the authors suggest that, with appropriate donor/recipient selection, suitable outcomes can be achieved. A higher MELD recipient and donor hemodynamic instability were associated with NAS and poorer graft survival[37].

HCV reinfection

The deleterious effect of donor age on the recurrence of HCV infection has been fully demonstrated. Berenguer *et al*[8] reported that the survival of transplant patients with HCV infection is decreasing, and aging donors is one of the main factors. Donor age is an independent factor associated with the risk of developing cirrhosis and decreased survival. Lake *et al*[38], using data from the American Scientific Registry of Transplant Recipients, analyzed the impact of donor age on the survival of 778 hepatitis B, 3463 hepatitis C, and 7429 non-viral recipients. In HCV-infected recipients, the strongest predictor of graft loss was donor age. Transplantation with organs from donors between ages 41 and 50, 51 and 60, and > 60 years old was associated with a linear increase in the risk of graft loss. Subsequent single or multicenter studies confirmed these findings[39-43]. Analysis of the Spanish Registry for Liver Transplantation presented a lower graft survival in HCV-infected patients when organs were procured from donors older than 50 years[43] (Figure 4). Ghinolfi *et al*[39] analyzed the use of octogenarian donors for LT. In those ⩾ 80 years old, the 5-year graft survival was lower for HCV-positive *vs* HCV-negative recipients (62.4% *vs* 85.6%, $P = 0.034$).

A correlation between accelerated fibrosis and worse outcome in grafts from older donors has been demonstrated[9,44]. Machicao *et al*[9] and Wali *et al*[44] reported that donors age 50 years or more had a median fibrosis progression rate of 2.7 units/year and time to cirrhosis of 2.2 years post-transplant. Donor age was also a strong factor in determining the likelihood of antiviral treatment success[45,46].

The impact on the LT outcomes of new direct-acting antiviral agents (DAA) against HCV has not been well established. These new drugs allow more simple treatment regimens and minimal toxicity, and when used in combination, achieve viral eradication

in most HCV patients who undergo treatment[47,48]. The high cost of DAA still limits treatment on a large scale in most countries. In the next decades, DAA may lead to a significant reduction in patients needing a liver transplant for HCV and improve graft survival rate by decreasing the reinfection rate after LT[49]. In HCV-positive recipients, the impact of donor age on LT outcomes may someday be the same as that if HCV-negative recipients.

LIVER REGENERATION AND AGING

Morphological and structural changes occur in the liver with aging. At the macroscopic level, the liver suffers a reduction in size and a decline in blood flow[50,51]. At the hepatocellular level, changes include a loss of the smooth endoplasmic reticulum, a loss in the number of mitochondria accompanied by an increase in their volume, an increase in the volume of the dense body compartment (secondary lysosomes, residual bodies, lipofuscin), and an increase in hepatocyte polyploidy[52]. Despite morphological changes, the performed clinical studies do not allow for the identification of important age-associated deficits in liver function, and it is generally assumed that the majority of liver functions are relatively well maintained with age[53].

Although age does not seem to significantly affect liver function, the incidence of several liver diseases increases with age whereas the capacity of the liver to manage or overcome liver diseases or external injuries decreases. In fact, the most dramatic and well-documented effect of aging in the liver is the impairment of liver regeneration. Hepatocytes are normally quiescent cells, but in response to liver injury, they can undergo extensive replication to restore the liver. This cellular transition from quiescence to proliferation requires activation of S-phase and mitotic-specific genes. However, fewer hepatocytes in elderly humans enter S-phase in comparison to younger people, and those that do are slower in doing so, compromising the rate of liver regeneration[53].

The loss of liver regenerative capacity is expressed by the decrease in cell cycle and the increase in autophagy and apoptosis[54]. However, despite these phenomena, reported over 50 years ago, the cellular and molecular basis for the loss of an aged liver's regenerative capacity has not been fully elucidated.

Different mechanisms have been suggested as implicated in the loss of this capacity with aging. Reduction in hepatocyte telomere length is one of these suggested mechanisms because it diminishes cell mitosis and apoptosis and thus produces a decline in cell proliferation. Takubo *et al*[55], after studying liver specimens from 94 individuals aged 0-101 years, found significant telomere shortening with age. Similar results were also observed in studies by Aikata *et al*[56] and Aini *et al*[57]. Hepatocytes presenting telomere shortening and karyotypic alterations were found in long-term transplanted human allografts. It appears that telomere shortening in liver cells is more significant in the early years, before the age of 40, when tissue turnover and growth are elevated[55,58]. This timing should be taken into consideration when comparing studies with controversial results because different donor age ranges were used.

Despite the clear connection between telomere shortening and reduction in cell proliferation, this association has not always implied impairment in liver regeneration. Experiments in a telomere restriction fragment-deficient mouse model demonstrated that liver regeneration after partial hepatectomy was not compromised by the loss of telomere integrity[59]. Post-hepatectomy regeneration was accomplished, increasing cell growth and yielding polyploid cells, indicating a switch from a proliferative to a cell growth pathway.

Another factor that suggests involvement of the decline in liver regeneration with aging is the inhibition of regeneration at an epigenetic level. Studies by Timchenko's group[60] indicate that the reduced proliferative response of aged livers is likely to be related to alterations in signal-transduction pathways (at the translational and/or post-translational levels). The decline in the regenerative capacity of old livers seems to be related to epigenetic silencing of E2F-regulated genes as a result of several age-dependent signal-transduction pathways. A decline in growth hormone with age leads to higher cyclin D3 levels that activate cdk4. Activated cdk4 promotes the formation of C/EBPα-Brm and CUGBP1-eIF2 complexes in livers of old mice. CUGBP1-eIF2 complexes up-regulate HDCA1 protein levels that, jointly with C/EBPα-Brm complexes, bind to E2F-dependent promoters, inhibiting expression of E2F-regulated genes and thus liver regeneration. In fact, it has been observed that treatment of old mice with growth hormone corrects liver proliferation[61].

In addition, hepatocellular response to growth factors has been proposed as another mechanism implicated in the reduction of liver regeneration with aging. The hepatocyte proliferative response to epidermal growth factor (EGF) is clearly increased in young rats compared to old animals, suggesting that aging impairs hepatocyte responsiveness to growth factors[53,60]. The problem does not seem to be related to the number of EGF receptors or their binding capacity but rather to a reduction in receptor phosphorylation, a critical step in the EGF-induced hepatocyte proliferation pathway[61].

Apart from the mechanisms mentioned previously, changes in the structure of hepatic sinusoidal endothelium, including a loss of fenestrae and a thickening of the endothelial cells (pseudo-capillarization), have also been associated with a decrease in liver regeneration with aging. Furrer *et al*[62] demonstrated that pseudo-capillarization contributes to age-related decline in regeneration after hepatectomy in mice. Their data demonstrated that treatment with a serotonin receptor agonist in old mice restored liver regeneration capacity

Table 3 Review of cellular and molecular mechanisms suggested to be implicated in the loss of aged liver's regenerative capacity

Mechanism	Ref.
Telomere shortening	Takubo *et al*[55] Aikata *et al*[56] Aini *et al*[57]
Transcriptional and post-transcriptional modifications	Timchenko[60] Wang *et al*[61]
Hepatocelullar response to growth factors	Schmucker[53] Wang *et al*[61]
Pseudo-capillarization	Furrer *et al*[62]
Decline of progenitor cell populations and changes in their niches	Ono *et al*[18] Yousef *et al*[64] Conboy *et al*[65] Wang *et al*[66]

through a vascular endothelial growth factor (VEGF)-dependent pathway. In their findings, the serotonin receptor agonist resulted in increased systemic VEGF availability, up-regulating the number and size of endothelial cell fenestrae, improving hepatic blood flow, and therefore enhancing the hepatic regenerative capacity. In this sense, higher VEGF secretion levels have also been detected in cultures of isolated human hepatocytes from young donors compared to those isolated from older donors[63].

Finally, a decline in the hepatic progenitor cell population has also been suggested as another possible cause of liver regeneration impairment in older donors. Ono *et al*[21] observed that the progenitor cell population (Thy-1$^+$) consistently tended to decline with age in LDLT. On the other hand, Yousef *et al*[64] recently found that the decline in stem cell function with age was largely due to biochemical imbalances in the cell niches, demonstrating that aging imposes an elevation in transforming growth factor β (TGF-β) signaling in the myogenic niche of skeletal muscle and in the neurogenic niche of the hippocampus. When they interfered with TGF-β levels by systemically decreasing TGF-β signaling with a single drug, bringing its levels closer to those detected in young mice, these authors could simultaneously enhance neurogenesis and muscle regeneration in the same old mice, findings further corroborated *via* genetic interference with TGF-β. Conboy *et al*[65] have previously reported similar observations in old mice permanently linked with their vascular systems (heterochronic parabiosis) to young mice. They reported a significant increase in proliferation of the aged hepatocyte progenitors in the old liver and restored expression of the complex c/EBP-alpha to levels seen only in young animals. Additionally, Wang *et al*[66] reported that senescent human hepatocytes can restore their proliferative capacity after xenotransplantation into mice, a finding with a potentially great impact on future studies of liver pathology and liver cell therapy. Hence, a process that once was thought to be terminal - *i.e.*, cell senescence and growth arrest - seems now to be tightly associated with the organ microenvironment rather than with the actual age of the organism. This relationship opens the door to the development of novel pharmacological strategies aimed at rejuvenating old liver grafts immediately after procurement and prior to transplantation.

Thus, understanding the cellular and molecular basis for the reduced proliferative response in old livers is important and could indicate how we can improve liver regeneration and graft survival in older patients. From this perspective, some studies have been designed to find specific markers to predict function and longevity of transplanted organs. Among those senescence markers that have been studied is the abovementioned telomere length; others include the senescence marker protein-30 (SMP-30), which has shown good results in animals that have not correlated with results in humans; CDKN2A/p16INK4a, which is a good predictor of long-term graft function in renal transplantation but has not yet been studied in liver models; the cyclooxygenases 1 and 2 (COX-1 and COX-2); the cell proliferation marker Ki-67; endoplasmic reticulum chaperone levels; and cytochrome p450 mRNA expression[67].

In old animals and in elderly humans liver regeneration is impaired, and it appears to be the rate of liver regeneration, rather than the regenerative capacity, that is diminished in the elderly (Table 3).

These age-related changes could be the factors that determine the higher sensitivity of the graft from older donor to develop irreversible lesions induced by distinct injuries, and results in higher rate of unsuitable response in older donor grafts.

CONCLUSION

The age of donors is increasing significantly in recent years, and liver grafts previously considered suboptimal because they came from elderly donors are nowadays used routinely in all centers. Although the various existing studies so far have contradictory results, age may have a role in the outcome of LT. The use of older donors has been linked to a greater number of biliary complications in both deceased and LDLT, as well as to a poor outcome of HCV recurrence injury. In addition, most LT prognostic scores have donor age as a fundamental variable. The pathophysiological bases of this association are not well established. Liver function does not seem to be influenced by aging, but several changes at the macroscopic and hepatocellular levels have been observed. There are also reported different biological changes in aging that lead to a loss of the liver's proliferative response and regeneration. These alterations may lead to an impairment of the capacity of the liver to manage and overcome liver diseases and to face external injuries.

Donor age is not the only relevant factor in the

outcome of LT, however; surgical factors such as ischemia time or hemodynamic instability during surgery, and recipient factors, such as MELD score, are also essential. Therefore, avoiding these factors as much as possible in liver transplants performed with elderly donors may lead to outcomes similar to those with transplants performed with younger donors.

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P- Reviewer: Bramhall S, Kim JH, Tanoglu A **S- Editor**: Gong ZM
L- Editor: A **E- Editor**: Ma S

World Journal of Gastroenterology

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DOI: 10.3748/wjg.v22.i21.4977

World J Gastroenterol 2016 June 7; 22(21): 4977-4987
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

REVIEW

Current status of laparoscopic and robotic ventral mesh rectopexy for external and internal rectal prolapse

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Author contributions: van Iersel JJ and Paulides TJC designed the study, performed the research, analysed the data and drafted the article; Verheijen PM, Lumley JW, Broeders IAMJ and Consten ECJ critically reviewed and revised the manuscript; all authors read and approved the final version of the manuscript.

Conflict-of-interest statement: No conflict of interest.

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Received: March 18, 2016
Peer-review started: March 21, 2016
First decision: March 31, 2016
Revised: April 15, 2016
Accepted: May 4, 2016
Article in press: May 4, 2016
Published online: June 7, 2016

Abstract

External and internal rectal prolapse with their affiliated rectocele and enterocele, are associated with debilitating symptoms such as obstructed defecation, pelvic pain and faecal incontinence. Since perineal procedures are associated with a higher recurrence rate, an abdominal approach is commonly preferred. Despite the description of greater than three hundred different procedures, thus far no clear superiority of one surgical technique has been demonstrated. Ventral mesh rectopexy (VMR) is a relatively new and promising technique to correct rectal prolapse. In contrast to the abdominal procedures of past decades, VMR avoids posterolateral rectal mobilisation and thereby minimizes the risk of postoperative constipation. Because of a perceived acceptable recurrence rate, good functional results and low mesh-related morbidity in the short to medium term, VMR has been popularized in the past decade. Laparoscopic or robotic-assisted VMR is now being progressively performed internationally and several articles and guidelines propose the procedure as the treatment of choice for rectal prolapse. In this article, an outline of the current status of laparoscopic and robotic ventral mesh rectopexy for the treatment of internal and external rectal prolapse is presented.

Key words: Laparoscopic ventral mesh rectopexy; Robot; Rectal prolapse; External rectal prolapse; Internal rectal prolapse; Rectocele; Mesh erosion; Obstructed defecation; Faecal incontinence; Biological mesh

Core tip: Globally, there is no uniformity for the treatment of internal and external rectal prolapse. Laparoscopic or robotic-assisted ventral mesh rectopexy is being progressively performed internationally for correcting rectal prolapse. This abdominal approach

avoids posterolateral rectal mobilization and the risks of an anastomosis, corrects the middle compartment, improves anorectal function and shows acceptable recurrence rates. In this article, a synopsis of the current status of laparoscopic and robotic ventral mesh rectopexy for the treatment of internal and external rectal prolapse is presented.

van Iersel JJ, Paulides TJC, Verheijen PM, Lumley JW, Broeders IAMJ, Consten ECJ. Current status of laparoscopic and robotic ventral mesh rectopexy for external and internal rectal prolapse. *World J Gastroenterol* 2016; 22(21): 4977-4987 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i21/4977.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i21.4977

INTRODUCTION

Prolapse of the posterior compartment of the pelvic floor, including rectal prolapse (RP) and its affiliated rectocele and enterocele, is associated with socially debilitating symptoms such as obstructed defecation, pelvic pain and faecal incontinence[1-3]. In the past decades, multiple surgical techniques have been described for RP. There is consensus that perineal procedures are associated with a higher recurrence rate and therefore, an abdominal approach, when possible, is preferred[4,5]. The majority of the abdominal procedures, however, include posterolateral mobilization of the rectum resulting in new-onset or worsening postoperative constipation[6].

In the search to reduce postoperative constipation, ventral mesh rectopexy (VMR) was developed[6]. In this procedure, the rectum is mobilized ventrally and attached to the sacral promontory with a mesh. By avoiding posterolateral rectal mobilization, autonomic nerves are spared and the risk of postoperative constipation is minimised. By lifting the middle compartment of the pelvic floor, correction of other frequently accompanying pelvic prolapses and celes is achieved.

Laparoscopic or robotic-assisted VMR is being progressively performed internationally and several articles and guidelines propose the procedure as the treatment of choice for RP[7-10]. This topic highlight summarises and assesses current evidence on laparoscopic and robotic ventral mesh rectopexy (LVMR/RVMR) for the treatment of internal and external rectal prolapse (IRP/ERP).

SELECTION OF USED LITERATURE

Studies presenting a homogeneous group of patients with rectal prolapse syndromes treated with VMR (laparoscopic or robotic) as described by D'Hoore *et al*[6], avoiding posterolateral mobilization and using a synthetic mesh were selected. Laparoscopic and robotic outcomes had to be displayed separately. Studies describing a heterogeneous group were excluded. Articles using a biological mesh are described separately.

COMPLICATIONS

Laparoscopic

Since the introduction of laparoscopic surgery, complications following rectopexy have reduced significantly[11]. Over the years, many studies have been published investigating surgical complications after LVMR. Most were small case series, but recently a large cohort of 919 patients with a median follow-up of 33.9 mo was published[12]. For this topic highlight, we have included 24 studies showing postoperative complication rates from 0% to 23.5% (Table 1). This extensive variation can be explained by the different ways in scoring morbidity between studies, especially for minor complications. Therefore, we have divided complications in minor and major groups according to the Clavien-Dindo (CD) classification[13]. Major complications, requiring surgical intervention (CD $\geqslant$ 3), are more relevant and in most cases directly ascribed to the VMR. Such complications were demonstrated from 0% to 7.7% of patients, which is acceptable and comparable to other minimal-invasive abdominal pelvic floor procedures[14]. Perioperative mortality is very low and occurred from 0% to 1.1%. Conversion is rare and was described from 0% to 5.9% with one study reporting a rate of ten percent. The majority of the conversions were due to extensive intra-abdominal adhesions.

Robotic

Three studies using synthetic mesh discussed the complication rate following RVMR (Table 1). Robotic surgery showed a non-significant minimal advantage in terms of intra- and postoperative complications compared to LVMR, described in a meta-analysis of these three studies[15]. However, studies were small and follow-up was short. Very recently, a randomised controlled trial (RCT) comparing the two techniques demonstrated a non-significant equality in complication rates[16].

SYNTHETIC MESH-RELATED COMPLICATIONS

Laparoscopic

The use of mesh in pelvic floor surgery has been subject for debate in recent years. Considerable commotion arose in response to the US Food and Drug Administration (FDA) report in 2011 where a high number of mesh-related adverse events associated with transvaginal pelvic organ prolapse repair were described[17]. The systematic review of Abed *et al*[18], showing a mesh erosion rate of 10.3% (110 studies, range 0%-29.7%) within 12 mo after transvaginal pelvic organ prolapse repair, confirmed these concerns.

Table 1 Conversion, intra- and postoperative complications following laparoscopic and robotic ventral mesh rectopexy with synthetic mesh *n* (%)

	n	Median FU (mo)	Intra-operative complications	Conversion	Postoperative complications			
					Total	Minor (CD 1-2)	Major (CD 3-4)	Mortality (CD 5)
Laparoscopic studies								
D'Hoore *et al*[6], 2004	42	61	0	2 (4.8)	2 (4.8)	2 (4.8)	0	0
D'Hoore *et al*[84], 2006	109	-	0	4 (3.7)	8 (7.3)	8 (7.3)	0	0
Slawik *et al*[62], 2008	80	54	-	1 (1.3)	7 (8.8)	7 (8.8)	0	0
van den Esschert *et al*[54], 2008	17	38[1]	0	1 (5.9)	4 (23.5)	3 (17.6)	1 (5.9)	0
Boons *et al*[44], 2010	65	19	-	1 (1.5)	11 (16.9)	6 (9.2)	5 (7.7)	0
Collinson *et al*[51], 2010	75	12	0	1 (1.3)	4 (5.3)	3 (4)	0	0
Wijffels *et al*[85], 2011	80	23	-	1 (1.3)	10 (12.5)	9 (11.3)	1 (1.3)	0
Wong *et al*[36], 2011	40	6	0	4 (10.0)	5 (12.5)	5 (12.5)	0	0
Wong *et al*[52], 2011	84	29	4 (4.8)	3 (3.6)	3 (3.6)	2 (2.4)	1 (1.2)	0
Lauretta *et al*[24], 2012	30	13.9[1]	-	0	2 (7.7)	0	2 (7.7)	0
Faucheron *et al*[86], 2012	175	74/60[2]	0	3 (1.7)	8 (4.6)	5 (2.9)	3 (1.7)	0
Formijne Jonkers *et al*[23], 2013	233	30	0	6 (2.6)	11 (4.7)	7 (3.0)	4 (1.7)	0
Badrek-Amoudi *et al*[25], 2013	48	33	-	0	9 (18.8)	8 (16.7)	1 (2.1)	0
Maggiori *et al*[26], 2013	33	42[1]	0	1 (3.0)	0	0	0	0
Mantoo *et al*[38], 2013	74	16	0	3 (4.1)	15 (20.0)	15 (20.0)	0	0
Mäkelä-Kaikkonen *et al*[37], 2014[3]	20	3	0	0	1 (5.0)	0	1 (5.0)	0
Mackenzie *et al*[32], 2014	953	21	-	8 (1.3)	63 (6.6)	53 (5.6)	8 (0.8)	2 (0.2)
Ogilvie *et al*[30], 2014	29	15.4	1 (3.4)	0	3 (10.3)	2 (6.9)	1 (3.4)	0
Randall *et al*[31], 2014	190	29	1 (0.5)	5 (2.6)	22 (11.6)	11 (5.7)	8 (4.2)	2 (1.1)
Owais *et al*[53], 2014	68	42	0	0	11 (16.2)	10 (14.7)	1 (1.5)	0
Gosselink *et al*[29], 2015	91	12	0	0	5 (5)	4 (4.4)	0	0
Tsunoda *et al*[33], 2015	26	16	0	0	2 (7.7)	2 (7.7)	0	0
Consten/van Iersel *et al*[12], 2015	919	33.9/120[2]	3 (0.3)	20 (2.2)	203 (23.4)	153 (19.3)	50 (4.1)	1 (0.1)
Tsunoda *et al*[34], 2016	31	25	0	0	2 (6.5)	2 (6.5)	0	0
Robotic studies								
Wong *et al*[36], 2011	23	6	0	1 (4.3)	1 (4.3)	0	0	0
Mantoo *et al*[38], 2013	74	16[4]	0	1 (2.3)	5 (11.0)	5 (11.0)	0	0
Mäkelä-Kaikkonen *et al*[37], 2014[3]	20	3	1 (5.0)	0	1 (5.0)	1 (5.0)	0	0

[1]Mean instead of median; [2]Percentages are Kaplan-Meier estimates at 60 and 120 mo of follow-up; [3]The results of Wong, Mantoo and Mäkelä-Kaikkonen *et al* are displayed per technique; [4]Not specified whether mean of median was used. FU: Follow-up; - : Not specified or not applicable.

The contemporary literature present a low incidence of mesh-related morbidity following VMR, but studies discussing this issue are limited. A pooled analysis of 11 observational studies (n = 767) demonstrated a 0.7% rate for mesh-related complications after LVMR in 2012[19]. Recently a multicentre study, including 2203 patients from databases of five hospitals over a 14-year period, described 45 patients (2%) developing mesh erosion (42 synthetic, 3 biologic) after a median of 23 mo[20]. However, underestimation is probable because of the retrospective character and a lack of systematic follow-up of this study. In general, mesh complication rates from 0% to 6.7% with mesh erosion percentages between 0 and 3.7% are described[12,19-34]. Most articles report vaginal mesh erosions, but intrarectal mesh migration following LVMR is not uncommon. The study of Evans *et al*[20] showed approximately half of the mesh erosions were rectal (17/45, 0.8%) and a similar percentage was described in other articles[20,27,31,35]. Recognized risk factors for developing mesh erosion are smoking, steroids, poorly regulated diabetes mellitus, pelvic hematoma, pelvic infection and a history of pelvic irradiation or pelvic surgery[19,35]. The multicentre study also suggested that mesh erosions were more frequently associated with LVMR for IRP (P = 0.02) and polyester mesh (P = 0.00006)[20].

Robotic

Only four studies mentioned examination for synthetic mesh-related complications following RVMR and all reported a rate of zero percent[36-39]. The follow-up, however, was short varying from 3 to 23 mo.

FUNCTIONAL OUTCOME

Laparoscopic - ERP

LVMR, with a limited anterior dissection, was introduced to avoid rectal denervation associated with damage to the parasympathetic fibres of the inferior hypogastric plexus. The RCT of Speakman *et al*[40] showed that preservation, rather than division, of the lateral ligaments is associated with less postoperative constipation. Meta-analyses confirmed this specific finding and demonstrate that VMR seems to be related to less constipation postoperatively as compared with other abdominal techniques (posterior mesh rectopexy, Ripstein, Orr-Loygue)[5,41].

An ERP is a circumferential full-thickness protrusion of the rectum through the anal verge. A recent consensus report, by a panel of international experts,

Table 2 Functional results following laparoscopic and robotic ventral mesh rectopexy with synthetic mesh

Laparoscopic studies	*n*	Median FU (mo)	Improvement OD	*P* value	Improvement FI	*P* value	Median gain CCCS	*P* value	Median gain CCIS	*P* value
Indication ERP										
D'Hoore *et al*[6], 2004	42	61	84.2%. *De novo* 4.8%	-	90.3%	-	-	-	13	< 0.001
Auguste *et al*[21], 2006	54	12	70%. *De novo* 17.6%		72.4%		-	-	5.8[1]	-
Verdaasdonk *et al*[42], 2006[2]	13	7	66%	-	69%	-	-	-	-	-
Cristaldi *et al*[43], 2007	63	18	78%	-	90%. *De novo* 3.2%	-	5	< 0.0001	32 (FISI)	< 0.0001
Boons *et al*[44], 2010	58[3]	19	72%	-	83%. *De novo* 1.5%	-	5	< 0.0001	36 (FISI)	< 0.0001
Formijne Jonkers *et al*[23], 2013[4]	36	30	57.9%	0.01	76.2%	< 0.001	-	-	-	-
Randall *et al*[31], 2014	190	29	-	-	93%	-	-	-	8	< 0.0001
Gosselink *et al*[29], 2015[4]	41	12	-	-	50%	< 0.01	4.8	< 0.01	12 (FISI)	< 0.01
Tsunoda *et al*[33], 2015[4,5]	19	12	52%	-	62%	-	7	< 0.0001	23 (FISI)	< 0.0001
Consten/van Iersel *et al*[12], 2015[4]	242	33.9	63.3%	< 0.0001	73.2%	< 0.0001	-	-	-	-
Tsunoda *et al*[34], 2016	31	12	-	-	-	-	5	0.005	22 (FISI)	< 0.0001
Indication IRP and/or rectocele										
Collinson *et al*[50], 2007	30	3	83%	-	92%	-	9	< 0.0001	25 (FISI)	< 0.0001
Collinson *et al*[51], 2010	75	12	86%	-	85%	-	7	< 0.0001	20 (FISI)	< 0.0001
Wong *et al*[52], 2011	84	29	45%	< 0.001	20%	> 0.05	-	-	-	-
Formijne Jonkers *et al*[23], 2013[4]	197	30	76.9%	< 0.001	65.4%	< 0.001	-	-	-	-
Gosselink *et al*[28], 2013	72	12	-	-	-	-	5	< 0.001	16 (FISI)	< 0.01
Gosselink *et al*[29], 2015[4]	50	12	-	-	48%	< 0.01	3.1	< 0.01	17 (FISI)	< 0.01
Tsunoda *et al*[33], 2015[4,5]	25	12	55%	-	63%	-	6	< 0.0001	22 (FISI)	< 0.0001
Tsunoda *et al*[87], 2015	26	16	-	-	-	-	7	< 0.01	24 (FISI)	< 0.01
Consten/van Iersel *et al*[12], 2015[4]	242	33.9	61%	< 0.0001	73.2%	< 0.0001	-	-	-	-
Indication both ERP and IRP and/or rectocele										
van den Esschert *et al*[54], 2008	1 ERP, 16 IRP	38[1]	-	-	-	-	+2.7[6] (ODS)	0.091	-	-
Lauretta *et al*[24], 2012	2 ERP, 28 IRP	13.9[1]	92.8%	-	85.7%	-	9.1 (ODS)[1][88]	< 0.05	7.1[1]	< 0.05
Badrek-Amoudi *et al*[25], 2013	11 ERP, 37 IRP	33	68%	< 0.0001	-	-	17 (ODS)[7][89]	< 0.0001	4	< 0.0001
Maggiori *et al*[26], 2013	33[8]	42[1]	72%. *De novo* 7%	-	90%	-	-	-	8	0.002
Mackenzie *et al*[32], 2014	149 ERP, 487 IRP	21	56.7%[9]. *De novo* 1.4%	0.119	89.7%[10]. *De novo* 1%	0.040	12 (ODS)[88]	-	8	-
Owais *et al*[53], 2014[11]	18 ERP, 50 IRP	42	82%	-	82%	-	12.5 (ODS)[90]	< 0.001	4	< 0.001
Robotic *vs* Laparoscopic studies - various indications										
De Hoog *et al*[39], 2009[1]	20 ERP *R*	23.4	-	-	-	-	3.2[1]	-	-	-
Mantoo *et al*[38], 2013[12]	23 ERP, 51 IRP *L*	16[13]	-	-	-	-	6 (ODS)[14]	0.004	4[14]	0.604
	12 ERP, 32 IRP *R*						14 (ODS)[14][91]	0.004	4[14]	0.604

[1]Mean instead of median; [2]One patient was excluded from further analysis, therefore *n* = 13 instead of *n* = 14 was used; [3]Functional data were complete in 58 of 65 patients; [4]The results of Formijne Jonkers *et al*, Gosselink *et al*, Tsunoda and Consten and van Iersel *et al* are displayed per indication; [5]Postoperative functional data were fulfilled in 44 of 59 patients; [6]Mean ODS score was 2.7 higher after surgery meaning function deteriorated postoperatively; [7]Pre- and postoperative ODS scores were available for 36 patients; [8]Of the 33 patients (ERP *n* = 20, *n* = 13 IRR) 3 lost to follow-up. For the remainder of patients the surgical indication was not given; [9]Based on 602 patients; [10]Based on 276 patients; [11]Only men included; [12]A modified version of the D'Hoore rectopexy used; [13]Not specified whether mean of median was used; [14]Estimation based on bar chart. OD: Obstructed defecation; FI: Faecal incontinence; ODS: Obstructed defecation syndrome score; L: Laparoscopic; R: Robot; RP: Internal rectal prolapse; ERP: External rectal prolapse; CCCS: Cleveland clinic constipation score; CCIS: Cleveland clinic incontinence score.

considers ERP a definitive indication for VMR[9]. LVMR showed improvement of obstructed defecation from 52% to 84.2% ($P < 0.01$-$P < 0.0001$)[6,12,21,23,33,42-44] with a median gain of the Cleveland Clinic Constipation Score (CCCS)[45] between 4.8 and 7 points ($P < 0.01$-$P < 0.0001$, Table 2)[29,33,34,43,44]. Obstructed defecation *de novo* was noted in 4.8% to 17.6% of patients[6,21]. Improvement of faecal incontinence was described in 50% to 93% of patients ($P < 0.01$-$P < 0.0001$)[6,12,21,23,29,31,33,42-44]. There was a median gain of the Cleveland Clinic Incontinence

Score (CCIS)[46] of 8 to 13 ($P < 0.001$ - $P < 0.0001$)[6,31] and one study reports a mean CCIS gain of 5.8 points[21]. The median Faecal Incontinence Severity Index (FISI)[47] benefit varied from 12 to 36 points ($P < 0.01$ - $P < 0.0001$)[29,33,34,43,44]. Two studies demonstrated new-onset faecal incontinence with an incidence of 1.5% and 3.2% in patients[43,44].

Laparoscopic - ERP and/or IRP and/or rectocele

An IRP is a telescopic infolding of the rectal wall during defecation. IRP is most commonly classified by the Oxford rectal prolapse grade differentiating between an intrarectal (grade 1 and 2) and intra-anal (grade 3 and 4) intussusception[48]. An Oxford grade 3 or 4 IRP, in combination with significant functional complaints failing to conservative therapy, is considered an indication for VMR[9,10]. The expert panel stated that VMR could also be performed for a complex rectocele of more than 3-4 cm[9]. However, a rectocele frequently exists with an IRP (80%) and, therefore, an isolated rectocele is rare (10%)[49]. LVMR for IRP and/or rectocele showed improvement of obstructed defecation from 55% to 86% ($P < 0.001$ - $P < 0.0001$)[12,23,33,50-52] with a median CCCS decrease between 3.1 and 9 points ($P < 0.01$ - $P < 0.0001$, Table 2)[28,29,33,34,50,51]. Improvement of faecal incontinence with 20% to 92% of patients ($P > 0.05$ - $P < 0.0001$)[12,23,29,33,50-52] and a median gain of FISI of 16 to 25 points ($P < 0.01$ - $P < 0.0001$) was observed in multiple cohorts[28,29,33,34,50,51]. None of the studies performing LVMR for IRP and/or rectocele described new-onset functional complaints.

Studies including both ERP and IRP and/or rectocele as indication for surgery showed 56.7% to 92.8% ($P = 0.119$ - $P < 0.0001$)[24-26,32,53] improvement for obstructed defecation complaints with a median advantage of 9.1 to 17 points in obstructed defecation syndrome (ODS) score ($P < 0.05$ - $P < 0.0001$, Table 2)[24-26,32,53]. One report described a non-significant deterioration in ODS score postoperatively[54]. A decrease in faecal incontinence complaints is reported from 82% to 90% of patients [24,26,32,53] with a median CCIS gain of 4 to 8 points ($P < 0.05$ - $P < 0.0001$)[24-26,32,53]. Literature demonstrated new-onset complaints of obstructed defecation between 1.4% and 7% of patients, with one report showing a *de novo* faecal incontinence rate of one percent[26,32].

Robotic

To date, only two studies using synthetic mesh discuss functional outcomes following RVMR (Table 2)[38,39]. The laparoscopic cohort of de Hoog *et al*[39] included various mobilizations and was excluded. The RVMR series of this study showed a median CCCS gain of 3.2 points, which was lower than other studies performing LVMR for ERP (Table 2). Mantoo *et al*[38], performing LVMR and RVMR for various indications, noted a significantly greater improvement for obstructed defecation after RVMR. Median gain of CCIS was non-significantly equivalent between the two techniques. Both improvement of obstructed defecation and faecal incontinence was in line with the literature on LVMR for various indications (Table 2). Functional outcome was not described in the recent RCT of Mäkelä-Kaikkonen *et al*[16]. However, this RCT did show a non-significant difference in postoperative residual rectoceles on MRI in favour of the robot compared with laparoscopy (8% *vs* 33%, $P = 0.26$). This may result in a better functional outcome for patients suffering obstructed defecation, but these outcome measures need to be evaluated at a longer follow-up.

RECURRENCE

Laparoscopic - ERP

With the introduction of minimally invasive surgery, recurrence rates with rectal prolapse surgery remained low and equivalent to those of open surgery[55,56]. In the nineties, three small trials suggested that preservation of the lateral ligaments might result in a higher recurrence rate[11]. Tou *et al*[11] speculated this was due to the limited mobilization of the rectum. Nonetheless, to date, numerous non-randomised observational studies, with increasing follow-up, quote acceptable recurrence rates following VMR. From 2004 until presently, recurrence percentages following LVMR for ERP range between 1.5% to 9.7%, with one small cohort ($n = 13$) reporting a rate of 15.4% (Table 3). Several reviews demonstrate comparable recurrence rates with various rectal mobilisations and abdominal techniques[5,22,41]. In addition, a multicentre, pooled analysis of 643 patients from 15 centres undergoing abdominal surgery for ERP, showed that the method of rectopexy did not influence the recurrence rate[57].

Variation in recurrence usually reflects differences of follow-up between studies. Articles reporting on LVMR for ERP described a time interval to presentation of recurrence between 10 and 91 mo after surgery. Most recurrences developed within the first 36 mo, but not all studies reported this time interval (Table 3). Little is known about risk factors for developing a recurrence following VMR. Mackenzie *et al*[32] found the only predictor of recurrence was the use of polyester mesh which generated a twofold increase in recurrence rate, with an odds ratio of 1.96 ($P = 0.017$), as compared with the most commonly used polypropylene graft.

Laparoscopic - ERP and/or IRP and/or rectocele

Three studies, performing LVMR for IRP and/or rectocele, quoted recurrence rates between 5.3 and 7.1 percent. Literature, including all rectal prolapse syndromes, reported recurrence percentages between 2.6% to 14.3%. The time interval between LVMR for various indications and recurrence varied from 10 to 139 mo (Table 3).

Table 3 Recurrence rates following laparoscopic and robotic ventral mesh rectopexy with synthetic mesh *n* (%)

Laparoscopic studies	*n*	FU (median)	Recurrence	Type of recurrence	Presentation of recurrence (mo)
Indication ERP					
D'Hoore *et al*[6], 2004	42	61	2 (4.8)	2 ERP	54, 91
Verdaasdonk *et al*[42], 2006[1]	13	7	2 (15.4)	2 ERP	-
Auguste *et al*[21], 2006	54	12	4 (7.4)	3 ERP, 1 IRP	26 (7-54)[2]
D'Hoore *et al*[84], 2006	109	-	5 (4.6)	4 ERP, 1 enterocele	-
Cristaldi *et al*[42], 2007	63	18	1 (1.7)	ERP	-
Boons *et al*[44], 2010	65	19	1 (1.5)	ERP	12
Wijffels *et al*[85], 2011	80	23	2 (2.5%)	2 ERP	6, 16
Faucheron *et al*[86], 2012	175	74/60[3]	2 (3)[3]	2 ERP	6, 24
Randall *et al*[31], 2014[5]	190	29	9 (4.7)	1 ERP, 8 IRP	25, 30, 31, 60[6]
Gosselink *et al*[29], 2015[3]	41	12	1 (2.3)	ERP	12
Tsunoda *et al*[34], 2016	31	25	3 (9.7)	3 IRP	10, 17, 31
Indication IRP and/or rectocele					
Collinson *et al*[51], 2010	75	12	4 (5.3)	4 IRP	-
Wong *et al*[52], 2011	84	29	6 (7.1)	6 rectocele	-
Gosselink *et al*[29], 2015[4]	50	12	3 (5.8)	3 IRP	-
Indication both ERP and IRP and/or rectocele					
Lauretta *et al*[24], 2012	2 ERP, 28 IRP	13.9[7]	1 (3.3)	1 IRP	19
Formijne Jonkers *et al*[23], 2013	36 ERP, 197 IRP	30	6 (2.6)	-	-
Badrek-Amoudi *et al*[25], 2013	11 ERP, 37 IRP	33	4 (8.3)	4 IRP	22 (median)
Maggiori *et al*[26], 2013	33[8]	42[7]	2 (6.7)	2 rectocele	11, 14
Mackenzie *et al*[32], 2014	149 ERP, 487 IRP	21	60 (9.4)	-	-
Owais *et al*[53], 2014[9]	18 ERP, 60 IRP	42	2 (2.9)	2 IRP	-
Consten/van Iersel *et al*[12], 2015	242 ERP, 677 IRP	33.9/120[3]	68 (14.3)[3]	15 ERP, 53 IRP	24.1 (1-139.4)[2]
Tsunoda *et al*[33], 2015	19 ERP, 25 IRP	26	2 (3.4)	2 IRP	10, 15
Robotic *vs* Laparoscopic - various indications					
De Hoog *et al*[39], 2009	20 ERP robot	23.4	4 (20)	-	-
Wong *et al*[92], 2011	23 IRP lap	12	1 (4.3)	Rectocele	3
	15 IRP robot		1 (6.7)	Rectocele	7
Wong *et al*[36], 2011	40 IRP lap	6	0 (0)	-	-
	23 IRP robot		0 (0)	-	-
Mantoo *et al*[38], 2013[10]	23 ERP, 51 IRP lap	16[11]	6 (8)	-	-
	12 ERP, 32 IRP robot		3 (7)	-	-
Mäkelä-Kaikkonen *et al*[37], 2014	14 ERP, 6 IRP lap	3	1 (5)	-	-
	13 ERP, 7 IRP robot		0 (0)	-	-

[1]One patient was excluded from further analysis, therefore *n* = 13 instead of *n* = 14 is used; [2]Mean (range); [3]Recurrence percentage is KM estimate at 60 and 120 mo of follow-up; [4]The results of Gosselink *et al* are displayed per indication; [5]Study group included the first 44 cases from Slawik *et al*[62]; [6]Only 4 time intervals are described; [7]Mean instead of median; [8]Of the 33 patients (ERP *n* = 20, *n* = 13 IRR) 3 lost to follow-up. For the remainder of patients the surgical indication was not given; [9]Only men included; [10]A modified version of the D'Hoore rectopexy used; [11]Not specified whether mean of median was used. Lap: Laparoscopic; IRP: Internal rectal prolapse; ERP: External rectal prolapse.

Robotic

The contemporary literature comparing LVMR with RVMR show similar recurrence rates between the two techniques (Table 3). Recurrence percentages vary from 0 to 7 for the robotic and 0 to 8 for the laparoscopic inclusions and were comparable to observational LVMR studies. One additional study from the de Hoog *et al*[39] noted a recurrence rate of 20% for the robotic, and 26.7% for the laparoscopic cohort. The laparoscopic series also included Well's procedures and therefore these results were excluded for analysis (Table 3).

MULTI-COMPARTMENT PROLAPSE

Pelvic floor dysfunction is regularly characterised by multi-visceral pelvic organ prolapse[58]. With an ageing population, the prevalence of uni- and multi-visceral pelvic organ prolapse will increase[59-61]. A growing number of articles discuss a multidisciplinary approach for multi-compartment prolapse, but only two studies avoid posterolateral rectal mobilization[62,63]. The first report, describing an open recto-vagino-vesicopexy, presented an improvement with constipation in 77% (P = 0.001), faecal incontinence in 69% (P = 0.005) and urinary incontinence in 50% (P = 0.18) of all patients respectively after 12 mo[63]. Two (8%) patients developed new-onset urinary incontinence. Slawik *et al*[62], performing a laparoscopic sacro-colpo-rectopexy, described an improvement in 91% of patients with faecal incontinence and a reduction in median CCIS of 10 points after six months. Obstructed defecation resolved in 80% of patients, but 7% of these underwent an additional bowel resection. New-onset obstructed defecation occurred in 3.8%, and urinary incontinence in 2.5% of patients respectively. No patient developed a recurrence after a median follow-up of 54 mo. Thus far, no robotic studies describing a

multi-compartmental approach with a limited anterior rectal dissection are published.

BIOLOGICAL MESH

Concerns over synthetic mesh-related complications such as erosion, dyspareunia, fistulation and stricturing have led to the introduction of a more expensive biological equivalent. The biological mesh is characterised by degradation of the graft and regeneration of host tissue[64]. In theory, this degradation could decrease the chance of erosion and chronic infection. Conversely, the partial resolution of the material may lead to a higher recurrence rate. In 2013, a systematic review by Smart *et al*[19] was published comparing 11 studies (767 patients) receiving synthetic mesh with two studies (99 patients) using a biologic graft. An erosion rate of less than one percent, with no difference identified between synthetic and biological mesh (0.7% *vs* 0%, P = 1.0%) was described. There was no significant difference in other mesh-related complications or short-term recurrence (3.7% *vs* 4.0%, P = 0.78). The multicentre study of Evans *et al*[20] and two recent biological mesh studies[65,66] (4 and 20 mo follow-up) showed similar rates of mesh erosion. However, Franceschilli *et al*[66] reported a much higher percentage prolapse recurrence rate of 14% after a mean follow-up of 20 mo. Improvement with obstructed defecation was described from 82% to 92% with a mean gain of CCCS between 9 and 13 points (P = 0.02 - P < 0.0001)[65-68]. Reduction in faecal incontinence complaints occurred in 73% and 85% of patients with a mean gain in FISI score between four and 6 points (P = 0.01-P = 0.001)[65-68]. One report demonstrated a median gain in CCIS of approximately 10 points (P = 0.0002)[65-68]. Wahed *et al*[67] was the only study describing new-onset complaints (4.6% with constipation and 3.1% with faecal incontinence). Only one study comparing and matching biological and synthetic mesh for LVMR (29 *vs* 29) excists, demonstrating no significant difference in mesh-related complications, recurrence or functional outcome after a median follow-up of 15.4 mo[30]. Mehmood *et al*[69], comparing 34 LVMR with 17 RVMR patients using biological mesh, demonstrated a minor significant advantage in median CCIS gain for LVMR (10 *vs* 9.5, P = 0.02). Conversely, a non-significant benefit in favour of the robot was seen in a reduction of the FISI (32 *vs* 35, P = 0.3). Both the functional outcomes of the robotic and the laparoscopic cohort compared favourably to other studies describing LVMR for ERP (Table 2). No recurrences or mesh-related complications were seen in either cohort after 12 mo. There is a lack of high-level comparative evidence with long-term follow-up for biological mesh, which demonstrates any significant difference in graft-related morbidity and recurrence rates. When more data becomes available, the choice of the mesh may be influenced by cost or possible comorbidity. In a recent publication a panel of experts suggested that biological grafts may be a better option in the following circumstances: young patients, women of reproductive age, diabetics, smokers, patients with a history of previous pelvic radiation or sepsis, inflammatory bowel disease, and in cases of intraoperative breach of the rectum or vagina[9].

DISCUSSION

LVMR has become popularised in the past decade and is the preferred technique for treating rectal prolapse syndromes by many surgeons, especially in Europe. The procedure is becoming increasingly applied with robotic assistance. The robot enhances visualisation and manoeuvrability to improve complicated procedures in the deep pelvis, such as dissection and intracorporeal suturing[70]. Robotic surgery has proven to be more expensive in the short-term, but may lead to an overall reduction of costs due to enhanced ergonomics for the surgeon[11,71,72]. However, a long-term cost-effectiveness analysis of LVMR *vs* RVMR has not been performed.

The current evidence shows that LVMR and RVMR are safe procedures in terms of intraoperative, post-operative and mesh-related complications. Both LVMR and RVMR generate an acceptable recurrence rate and satisfactory improvement of functional outcome, with only one small laparoscopic cohort reporting an overall non-significant deterioration with obstructed defecation after surgery[54]. There may be a trend towards a better outcome for obstructed defecation following RVMR as compared with LVMR, but the level of evidence is low[16,38]. LVMR and RVMR show similar good results for improvement of faecal incontinence. Based on the currently available data, no superiority for either technique can be determined. As compared with other observational studies describing an abdominal approach to treat rectal prolapse syndromes, VMR shows similar recurrence rates and less constipation postoperatively[5,22,41]. Circumspection is required interpreting these results, however. Heterogeneity between the articles in patient selection and outcomes measured makes it difficult to draw conclusions from the current literature. In addition, follow-up has been relatively short and lacks a systematic approach for the majority of studies, especially the robotic series. Since pelvic floor dysfunction increases with age, long-term follow-up is required to assess functional outcome and recurrence. The true mesh erosion rate can only be obtained with adequately powered, long term studies incorporating a vaginal and anorectal examination for every patient. Thus far, only level 3 evidence exists with a paucity of RCT's and case controlled trials. There are no results of comparative studies including VMR available. In a recent critical appraisal, Lundby and Laurberg expressed their concerns about the rapid implementation of LVMR for obstructed defecation syndrome based on the contemporary evidence[73].

High-level comparative evidence is necessary to overcome these doubts and to determine the value of VMR in the definitive treatment of rectal prolapse syndromes.

FUTURE RESEARCH

This review focusses solely on VMR, but more than three hundred different procedures to treat rectal prolapse syndromes have been described. Thus far, no technique has been shown to be superior. This was confirmed by an international survey in 2012, showing no uniformity of surgical procedure[74]. The survey demonstrated, inter alia, that more than 20% of the surgeons preferred stapled transanal resection of the rectum (STARR) for the treatment of IRP. Festen *et al*[75] suggested an IRP associated with fecal incontinence should be treated with LVR and an IRP in combination with obstructed defecation with STARR or LVR. At present, one Italian trial is comparing LVMR with STARR for obstructed defecation syndrome[76]. In addition, there are eight ongoing surgical trials, of which five are mentioned by the cochrane study of Tou *et al*[11]; two comparing LVMR with Delorme's procedure for ERP[77,78], one investigating the outcomes of LVMR versus laparoscopic posterior rectopexy without mesh[79], one comparing laparoscopic resection rectopexy (RR) with laparoscopic fixation rectopexy[80], one assessing the difference between standard mesh rectopexy with ventral rectopexy[81], one studying the efficacy of LVMR for the treatment of chronic constipation[82] and two examining LVMR versus RVMR[16,83]. The trial by Mäkelä-Kaikkonen *et al*[16] has presented its short-term results, but long-term outcomes are awaited. The survey also shows VMR and RR are the two most common abdominal procedures for RP[74]. RR was developed to reverse the symptoms of rectal denervation inertia which is associated with traditional posterolateral rectal mobilization, but introduces the risks of a pelvic anastomosis. Three trials, comparing (predominantly open) abdominal rectopexy with and without sigmoid resection, described that RR was associated with less postoperative constipation but with a higher complication rate ($P > 0.05$)[11]. There is a need for a well-designed and adequately powered RCT comparing these two techniques laparoscopically or robotic-assisted. Lastly, high-quality evidence for the choice of a specific mesh type, either synthetic or biological, is required. The authors do acknowledge the slow recruitment and logistical difficulties of performing such trials, however.

CONCLUSION

Ventral mesh rectopexy (laparoscopic and robotic) appears a safe and effective procedure to correct different rectal prolapse syndromes with a low morbidity rate, acceptable long-term recurrence rates and a good functional outcome.

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P- Reviewer: Asteria CR, Smart NJ, Wasserberg N
S- Editor: Gong ZM **L- Editor**: A **E- Editor**: Wang CH

World Journal of Gastroenterology

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DOI: 10.3748/wjg.v22.i21.4988

World J Gastroenterol 2016 June 7; 22(21): 4988-4998
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

ORIGINAL ARTICLE

Basic Study

Multiorgan chronic inflammatory hepatobiliary pancreatic murine model deficient in tumor necrosis factor receptors 1 and 2

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Author contributions: Oz HS designed and executed the study, prepared the paper and provided funding; author has approved the final paper.

Supported by The NIH grant R03-DE019177.

Institutional animal care and use committee statement: All animal procedures were approved by the University of Kentucky Institutional animal care and use committee.

Conflict-of-interest statement: Author declares to have no commercial or associative interest that represents a conflict of interest in connection with the work submitted.

Data sharing statement: No additional data are available.

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Received: January 15, 2016
Peer-review started: January 18, 2016
First decision: February 18, 2016
Revised: March 21, 2016
Accepted: April 7, 2016
Article in press: April 7, 2016
Published online: June 7, 2016

Abstract

AIM: To provoke persistent/chronic multiorgan inflammatory response and to contribute to stones formation followed by fibrosis in hepatobiliary and pancreatic tissues.

METHODS: Tumor necrosis factor receptors 1 and 2 (TNFR1/R2) deficient mice reared in-house were given dibutyltin dichloride (DBTC) twice within 10 d by oral gavage delivery. Sham control animals received vehicle treatment and naïve animals remained untreated throughout the study. Animals were monitored daily for symptoms of pain and discomfort. The abdominal and hindpaw hypersensitivity were assessed with von Frey microfilaments. Exploratory behaviors were recorded at the baseline, after initiation of treatment, and before study termination. Histopathological changes were examined postmortem in tissues. Collagen accumulation and fibrosis were confirmed with Sirius Red staining.

RESULTS: Animals lost weight after oral administration of DBTC and developed persistent inflammatory abdominal and hindpaw hypersensitivity compared to sham-treated controls ($P < 0.0001$). These pain related secondary mechanical hypersensitivity responses increased more than 2-fold in DBTC-treated animals. The drastically diminished rearing and grooming rates persisted after DBTC administration throughout the study. Gross as well as micropathology at one month confirmed that animals treated with DBTC developed chronic hepatobiliary injuries evidenced with activation of stellate cells, multifocal necrosis, fatty degeneration

of hepatocytes, periportal infiltration of inflammatory cells, and prominent biliary ductal dilation. The severity of hepatitis was scored 3.7 ± 0.2 (severe) in DBTC-treated animals *vs* score 0 (normal) in sham-treated animals. Fibrotic thickening was extensive around portal ducts, in hepatic parenchyma as well as in lobular pancreatic structures and confirmed with Sirius Red histopathology. In addition, pancreatic microarchitecture was presented with distortion of islets, and parenchyma, infiltration of inflammatory cells, degeneration, vacuolization, and necrosis of acinar cells and distention of pancreatic ducts. Extent of pancreatic damage and pancreatitis were scored 3.6 ± 0.4 (severe) for DBTC-treated in contrast to score 0 (normal) in sham-treated animals. The gall bladder became expanded with ductal distention, and occasional bile stones were detected along with microscopic hepatic lesions. DBTC-treated animals developed splenic hypertrophy with increased weight and length ($P < 0.01$) along with thymic atrophy ($P < 0.001$). Finally, colitic lesions and colitis were prominent in DBTC-treated animals and scored 3.4 ± 0.3 (moderately severe) *vs* 0 (normal) for the sham-treated animals.

CONCLUSION: This is the first report of chronic inflammatory multiorgan hepatobiliary pancreatitis, along with fibrosis and calculi formation induced reliably utilizing oral DBTC administration in TNFR1/R2 deficient mice.

Key words: Inflammatory pain; Multiorgan; Hepatitis; Pancreatitis; Calculi formation; Gall bladder; Hepatobiliary inflammation

Core tip: Currently there is no reliable model for chronic multiorgan inflammatory and fibrosis. Tumor necrosis factor (TNF)α initiates inflammation through TNFR1/R2. TNFR1/R2 deficient mice administered orally with dibutyltin dichloride (DBTC) developed significant persistent inflammatory and pain related secondary mechanical hypersensitivity. DBTC-animals showed severe chronic hepatobiliary injuries and prominent biliary ductal dilation. Extensive fibrotic thickening was evidenced around portal ducts, in hepatic and pancreatic structures. DBTC-animals had severe pancreatic damage and pancreatitis, hepatic lesions with expansion of gall bladder, bile stones and severe colitis. This is the first report of chronic inflammatory multiorgan hepatobiliary pancreatitis, fibrosis and calculi formation in TNFR1/R2 deficient mice.

Oz HS. Multiorgan chronic inflammatory hepatobiliary pancreatic murine model deficient in tumor necrosis factor receptors 1 and 2. *World J Gastroenterol* 2016; 22(21): 4988-4998 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i21/4988.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i21.4988

INTRODUCTION

Fibrogenesis is a required process in wound healing, but persistent inflammatory and fibrotic reaction can lead to devastating symptoms and eventually organ failure[1,2]. Multiorgan fibrosis is typically the end product of various unresolved or repetitive tissue injuries from chronic inflammation, infection, radiation exposure, and abnormal repair outcome. Loss of function contributes to progression of morbidity and mortality. Multiorgan fibrosis is a common complication in cystic fibrosis[3], systemic sclerosis[4] and primary sclerosing cholangitis[5]. Chronic pancreatitis, initiated by idiopathic or recurrent inflammation, is manifested with irreversible destruction of exocrine parenchyma and pancreatic fibrosis. It is a potentially fatal progressive disease leading to diabetes mellitus and pancreatic cancer. Pancreatitis is associated with spontaneous visceral pain as a chief symptom in patients. Neural innervation of the pancreas is pivotal in the instigation and continuation of inflammation and pain response. Cellular destruction leads to activation of pancreatic sensory neurons causing release of neurotransmitters in the spinal cord and neurogenic signaling then back to the pancreas provoking plasma extravasation and neutrophil infiltration[6].

Multifactoral gallstones are one of the most prevalent gastrointestinal complications with serious outcomes such as gallstone pancreatitis and cancer. Gallstone disease is a chronic recurrent hepatobiliary complication which is characterized by formation of gallstones in the hepatic and bile duct, or gallbladder. It is manifested by impaired metabolism of cholesterol, bilirubin and bile acids[7].

Tumor necrosis factor α (TNFα) a proinflammatory cytokine, up-regulates various cytokines/chemokines to initiate acute and chronic stages of inflammation. The biological action of TNFα is chiefly through two gene family receptors, TNFR1 and TNFR2. TNFα is released mainly by activated macrophages, in addition to astroglia, microglia, CD4+ lymphocytes, Natural killer cells (NK), and neurons[8-10]. The complete-length membrane-crossing TNFα (mTNFα) is sliced by the inducible TNF converting enzyme (TACE) to release soluble TNFα (sTNFα) and diffusible peptide[11]. TNFα release is associated with inflammatory response and pain related sensation in patients with pancreatitis, hepatitis and inflammatory bowel disease (IBD), as well as neuropathy[12]. TNFα contributes to development of neuropathic pain[13]. Soluble TNFR1 and R2 neutralize circulating TNFα to alleviate pain related responses to mechanical allodynia, thermal hyperalgesia or peripheral nerve injuries[14-16]. TNFα plays an important function in the pathogenesis of acute pancreatitis. Recent investigations have demonstrated that TNFα inhibition drastically ameliorates the duration of experimental acute pancreatitis[17]. TNFα receptor 1 (TNFR1) gene deletion and etanercept application likewise ameliorated the duration of acute pancreatitis

in animal models, suggesting potential of etanercept and anti-TNFα monoclonal antibodies as therapy in clinical pancreatitis[17]. Although, current clinical treatments with these biological agents may diminish inflammation and pain by reducing TNFα and other cytokines, the inflammatory response and pain is likely to re-emerge in most patients with autoimmune disease including arthritis and IBD[10]. In addition, anti-TNFα monoclonal antibodies therapy has potential side effects such as provoking infections with JC virus, fungi and tuberculosis. Currently there is no cure or reliable mouse model for chronic pancreatitis.

Previously we have demonstrated that the baseline mechanical and thermal response to noxious stimulation is similar in TNFR1/R2 deficient mice *vs* wildtype background mice. However, TNFR1/R2 deficient mice develop more severe responses when similarly treated with various insults[10,16]. Animal models of acute and chronic pancreatitis have been utilized to examine mechanisms of pathogenesis, and to test possible therapeutic interventions. One of the most commonly used pancreatitis models is created by serial intraperitoneal administration of concentrations of caerulein, an ortholog of cholecystokinin[17]. Other chemically induced models have utilized di-n-butyltin dichloride (DBTC). DBTC is a polyvinyl carbonate (PVC) plastic stabilizer/catalyzer additive, insecticide and biocide in agriculture, and antifouling agent in the paint and fabric industry that often contaminates food and water[18]. Tail vein slow injection of DBTC induces relatively unpredictable pancreatitis flares in rats[6]. However, DBTC injection is tedious and minor leakage results in tail necrosis, gangrene and animal loss. We hypothesized that oral administration of DBTC would provoke persistent and chronic pancreatitis in animals deficient in TNF-receptors. Similarly, TNFR1/R2 may accelerate inflammatory response in multiorgans and contribute to stones formation and fibrosis in hepatobiliary and pancreatic tissues. Here we report a chronic persistent DBTC-induced inflammatory model by oral gavage in TNFR1/R2 deficient mice persisting at least one month allowing more clinically relevant studies in this model. Pain related behaviors accompanying this model are characterized.

MATERIALS AND METHODS

Animals

All animal procedures were approved by the University of Kentucky Institution Animal Care and Use Committee (IACUC). Mice were monitored daily for continued weight gain/loss and general health. Health status and procedures were documented daily on the UK IACUC Standard Operating Procedure (SOP-102) Post-Operative Evaluation form. Experiments were performed using dually deficient TNFR1/R2 mice (Jackson Laboratory) on a B6129SF2/J background inbred at the University of Kentucky animal facilities and provided by Dr. Westlund. Mice were housed in individual cages with a 10 h/14 h dark/light reversed cycle to accommodate behavioral test during their active dark period. Mice were allowed free access to food and water ad libitum, except 2 h before and during behavioral testing.

Induction of persistent chronic pancreatitis

Chronic persistent pancreatitis was induced in mice utilizing DBTC (Dibutyltin dichloride, Sigma-Aldrich, St Louis, MO). DBTC (10 mg/kg) was dissolved in 95% ethanol (two parts) and then mixed with glycerol (three parts) and given orally. Mice received DBTC by oral gavage (200 μL volume). Intragastric gavage administration was performed by Dr. Oz, an expert veterinarian scientist, in conscious animals, using appropriate bended gavage needles (22 gauge, 1 inch length, 1.25 mm ball diameter). Sham control mice were given the vehicle (95% ethanol + glycerol, 2:3) alone and Naïve control animals remained untreated. Animals were monitored until fully active. In order to induce chronic inflammation, mice received a 2nd treatment by oral gavage within 10 d. Following induction of pancreatitis the animals were monitored daily for activity, appearance, and signs of abdominal discomfort. They were weighed regularly and tested for hypersensitivity on the hindpaw plantar foot pad and the shaved abdominal surface. After completion of the final behavioral testing, one month after induction, the animals were euthanized with isoflurane overexposure, the thorax opened, blood samples collected by cardiac puncture, and tissue samples collected for histological evaluation.

Assessment of secondary mechanical allodynia by testing hindpaw withdrawal threshold

Pain-related behavior was assessed throughout the study by the determining secondary mechanical threshold to assess hyperalgesia/allodynia. The von Frey test is a standard comparison used in the field of pain research. Day 0, baseline testing to determine footpad nociceptive responses was performed testing hindpaw withdrawal latency to mechanical stimuli with von Frey fibers. Reflex testing for secondary mechanical hyperalgesia/allodynia with von Frey fibers was developed by Max von Frey, who in 1896 identified "pain spots" on human skin. Mechanical nociceptive thresholds were analyzed as described previously[10,19]. Paw withdrawal response latencies were assessed weekly throughout the study. Mice were placed into clear cylindrical plastic enclosures (7 cm × 4 cm × 4 cm) on a smooth metal meshed (3 mm × 3 mm) platform (36 cm × 29 cm × 21.5 cm). Mechanical withdrawal threshold testing was done on the plantar surface of both hindpaws using a set of 8 von Frey monofilaments [(4.74) 6.0 g; (4.31) 2.0 g; (4.08) 1.0 g; (3.61) 0.4 g; (3.22) 0.16 g; (2.83) 0.07 g; (2.36) 0.02 g; (1.65) 0.008 g]. The von Frey

filaments were applied perpendicularly to the plantar surface with sufficient force to bend the monofilament slightly and held for about 5 s, and 5 to 10 times with 15 s intervals. A positive response was defined as an abrupt withdrawal (flick response) of the foot during stimulation or immediately after the removal of stimulus. Whenever there was a negative or positive response, the next stronger or weaker filament was applied, respectively. Testing proceeded in this manner until four fibers had been applied after the first one caused a withdrawal response, allowing the estimation of the mechanical withdrawal threshold.

Pain-related behavioral evaluations for abdomen: Prior to induction of inflammation with DBTC administration, baseline testing of abdominal nociceptive responses to mechanical stimuli was performed with von Frey fibers applied to the upper left abdominal quadrant skin of mice as previously described[10,19]. Mechanical hypersensitivity in the abdominal area was quantified by measuring the number of withdrawal events (either abdominal withdraw from the von Frey filament or consequent licking of the abdominal area, or whole body withdrawal) in response to normally innocuous or sub-threshold mechanical stimuli. Testing continued weekly throughout the study.

Evaluation of the pain-related posture: The abnormal posture of each animal with an affected hindlimb was given a single score using a subjective pain-related behavioral scale (spontaneous pain rating score 0-5) *i.e.* 0- normal; 1- curling of the toes, 2- aversion of the paw; 3- partial weight bearing; 4- non-weight bearing and guarding; and 5- avoidance of any contact with the hindlimb.

Pain-related gait disturbance: Gait disturbances (curling toes, limping, guarding, rearing and grooming were tallied by an observer blinded to treatment group as in our previous studies[10].

Spontaneous visceral pain assessment: The animals were placed individually in the observation chamber for a 25 min recording session. The observation chamber is a 28 cm × 17.5 cm × 12.5 cm see-through plastic home cage with one mirrored side located in an isolated room with constant "white noise". A digital camera located 0.5 meter from the chamber with an unobstructed view was used to record animals spontaneous visceral pain related behaviors. The camera was linked to a computer recording program for offline data analysis (Logitech Image Studio). The chamber was washed with a detergent disinfectant and dried after each use between animals. Postures defined as statistically significant increase in visceral pain-related behavior in this study included rearing, grooming and licking of the lower abdomen, stretching the abdomen or hindlimb, lowering the abdomen against the floor, and abdomen retractions or arching the back. Recordings were masked and analyzed by the investigator.

Necropsy and sample collection

Tissue collection: At the end of the one month experiment, animals were deeply anesthetized with isoflurane inhalation. Pancreatic, hepatic, gall bladder tissues were excised and a portion was fixed in cold 4% paraformaldehyde in 0.1 mol/L phosphate buffer saline (PBS). Thymus and splenic tissues were removed, weighed, and fixed in paraformaldehyde. Colonic tissues were removed and flushed with cold PBS, and portion of ascending and descending colon were fixed for histopathological examinations.

Histopathology

Hepatic and pancreatic samples were collected and immerse fixed overnight in 4% paraformaldehyde in 0.1 mol/L PBS, then transferred into 70% ethanol and embedded in paraffin. Sections were cut (5 μm), rehydrated, stained with hematoxylin and eosin for histopathological changes. In order to detect collagen fiber deposits, sections were further stained with Sirius Red (Electron Microscopy Sciences, #26357-02), using routine histological protocols[20].

Pancreatitis scores

Pancreatic tissues and a portion of the small intestine along with spleen were removed and processed for histopathological evaluations of the pancreatitis. The severity of lesions was scored on a 0-4 grade on the basis of the histopathological changes as follows: 0 - normal pancreatic microstructure, no inflammatory mononuclear cell infiltration; 1 - slight inflammatory mononuclear cell infiltration, with no detectable parenchymal destruction; 2 - mild pancreatitis, edema, focal parenchymal destruction with mononuclear cell infiltration; 3 - moderate pancreatitis, with diffuse parenchyma destruction, presence of necrosis, and reduced number of islets; and 4 - severe pancreatitis, parenchyma mostly destroyed and replaced with adipose tissues, loss of pancreatic islets, presence of fibrosis and or calculi.

Hepatitis score

A portion of the right lobe from liver tissues of each mouse was placed in an embedding cassette and fixed in paraformaldehyde as mentioned above. The specimens were dehydrated and embedded in paraffin, and tissue sections of 5 μm were stained with Hematoxylin Eosin. Each slide was evaluated under Ziess light microscopy. Hepatic lesions were graded on a scale of 0 to 4+ based on degeneration, inflammation, and necrosis as follow: Grade 0 - no detectable lesions, degeneration, infiltration of inflammatory cells, normal tissue appearance; Grade 1 - focal infiltration of inflammatory cells in the tissue and

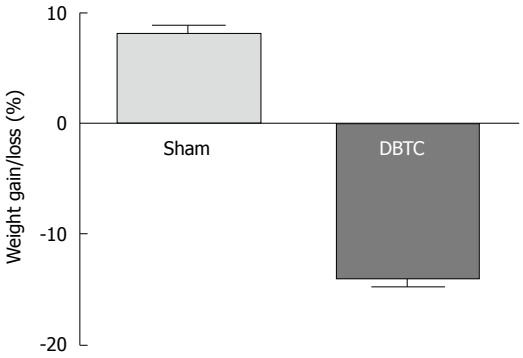

Figure 1 Percent weight gain in sham-treated controls (Sham) *vs* weight loss in dibutyltin dichloride-treated animals. *n* = 8 animals/group (*P* < 0.001). DBTC: Dibutyltin dichloride.

hepatocytes degeneration; Grade 2 - mild multifocal infiltration of inflammatory cells, and hepatocytes degeneration; Grade 3 - moderate multifocal infiltration of inflammatory cells and hepatocytes degeneration; and Grade 4 - severe diffuse infiltration of inflammatory cells, necrosis, or fibrosis.

Colitis evaluation scores

Colonic tissues were flushed with PBS (pH 7.2) and a portion from proximal and distal colonic tissues were fixed for histological examinations. The fixed sections were processed and stained with Hematoxylin Eosin and slides evaluated by Ziess light microscopy. The severity of colitis was assessed with a histological semi-quantitative grading score. The scores were based on histopathological features with a numeric value (0: normal to 4: severe) assigned according to the tissue involvement corresponding to the following criteria[21,22]. Grade 0: No detectable lesions, no inflammatory cells, and normal mucosal appearance; Grade 1: Focal inflammatory infiltrate in the mucosa; Grade 2: Mild multifocal inflammation with moderate expansion into the mucosa; Grade 3: Moderate multifocal inflammation with moderate expansion of the mucosa; and Grade 4: Severe diffuse inflammation with crypt epithelium disruption and ulceration.

Statistical analysis

All results are expressed as mean and standard error of mean (± SEM) unless otherwise stated. Data were analyzed using paired *t*-test comparison of groups for histology or analysis of variance (ANOVA) followed by Bonferroni post hoc comparison using GraphPad Prism Software for behavioral testing over time (San Diego, CA, United States). Statistical significance was set at $P \leqslant 0.05$.

RESULTS

Body weight loss

No major differences in body weight, behavioral analysis was detected between sham-treated and naïve control animals. Additionally, sham-treated and naïve control animals and wildtypes did not develop any histopathological lesions. Therefore, only sham-treated controls are reported here. Oral administration of DBTC resulted in weight lost as early as 3 d after treatment which persisted throughout the study, and animals developed persistent inflammatory abdominal and hindpaw hypersensitivity as compared to sham-treated animals. DBTC application induced significant body weight loss ($P < 0.001$) in compression to weight gain in sham-treated control animals. The major weight loss occurred during the 1st wk of DBTC inoculation when animals lost about 10% of their body weight, compared to weight gain in sham-treated animals. The body weight afterward became stable in DBTC-treated mice until the end of the one month study, but remained less than the sham-treated group (Figure 1).

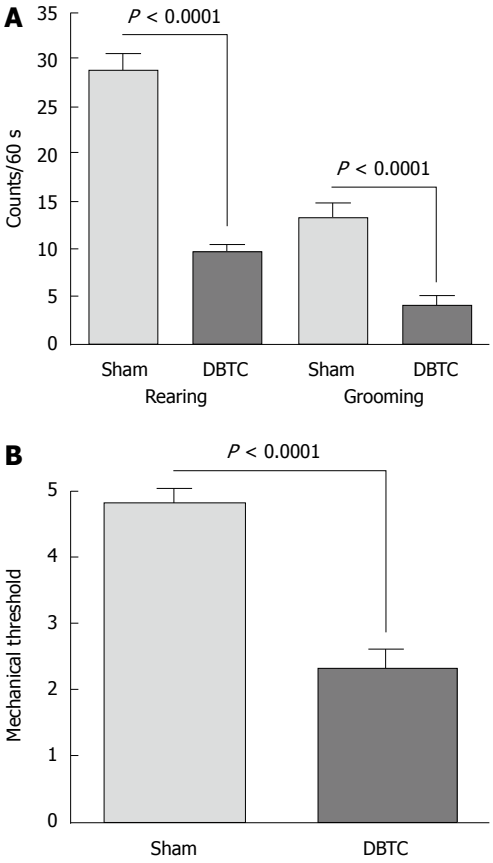

Figure 2 Rearing and grooming rate changes and hindpaw mechanical threshold modification due to dibutyltin dichloride-treatments. A: Rearing and grooming rates significantly diminished in dibutyltin dichloride (DBTC)-treated animals *vs* the sham-administered animals and persisted throughout the study (*P* < 0.0001); B: Hindpaw withdrawal threshold response to mechanical stimuli in DBTC-treated animals (DBTC) had significant decreased *vs* sham-administered control (sham) mice (*P* < 0.0001). *n* = 5 animals/group.

Behavioral pain related modifications

DBTC-treated animals had significant reduction in physical activities such as, cage crossing, rearing and grooming activity ($P < 0.0001$) compared to

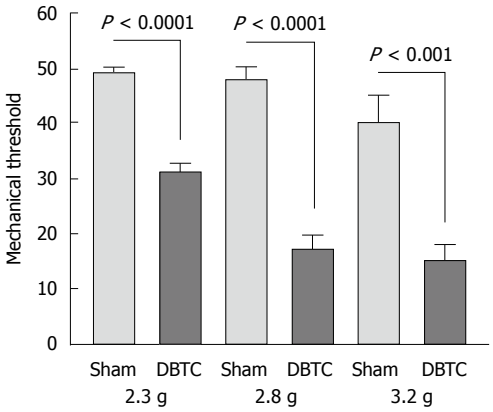

Figure 3 Abdominal threshold sensitivity to mechanical stimuli was determined with different gram forces of von Frey microfilaments as demonstrated here to 2.3 g ($P < 0.001$), 2.8 g ($P < 0.0001$), 3.2 g ($P < 0.001$), in dibutyltin dichloride-treated *vs* sham-treated controls (sham). Data is presented as the 50% mechanical threshold response. $n = 5$ animals/group.

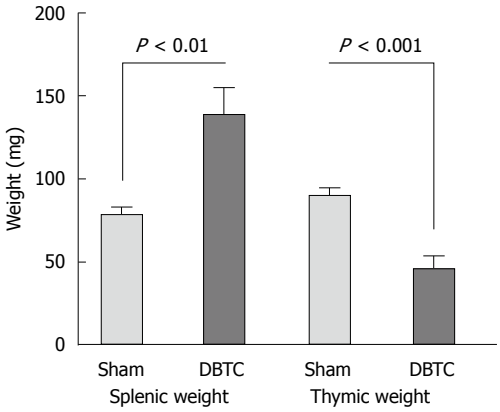

Figure 4 Splenic weight as well as splenic length (not shown) significantly increased in dibutyltin dichloride-treated animals ($P < 0.01$). In contrast thymic weight decreased in dibutyltin dichloride-treated (DBTC) *vs* the sham-treated (sham) animals ($P < 0.001$) due to atrophy and depletion of lymphocytes and thymocytes. $n = 8$ animals/group.

naïve sham-treated ones presumably attributed to the abdominal discomfort (Figure 2A). Hindpaw mechanical threshold was significantly decreased in DBTC-treated animals ($P < 0.0001$) tested using von Frey microfilaments (Figure 2B). In addition, responses to 3 different von Frey fibers with increasing grams force applied to the abdominal skin indicated DBTC-treated animals had significantly decreased mechanical threshold compared to sham-treated control (respectively $P < 0.0001$ from force 2.3 g, 2.8 g, $P < 0.001$ to force 3.22 g) (Figure 3). In contrast sham-treated animals demonstrated a partial visceral response to the higher filaments with forces of 3.22 g and above.

Splenomegaly and thymic degeneration

DBTC animals developed splenic hypertrophy with significant increase in weight and length of the spleens ($P < 0.01$). Splenic histopathologic studies demonstrated loss of medulla, irregular formation of trabecules, with captured trace of blirubin and bile deposits. In contrast, thymic tissues from DBTC-treated animals showed central degeneration and atrophy. The thymus was atrophied, and thymic weight significantly decreased in DBTC-treated animals ($P < 0.001$) compared to sham-treated animals (Figure 4).

Pancreatitis

Sham-treated animals demonstrated normal pancreatic structures with prominent islets (Figure 5A). In contrast, DBTC-treated animals developed gross as well as micropathology confirming the moderate to severe chronic pancreatitis. Pancreatic parenchyma presented with edema, congestion, distortion of microarchitecture, and infiltration of inflammatory cells. Pancreatic and acinar cells showed degeneration, fatty necrosis, and fibrosis. The pancreatic ducts became prominent and distended in DBTC-treated animals. These findings were consistent with fibrotic thickening which were particularly prominent in the vicinity of the primary duct, as well in surrounding lobular pancreatic parenchyma as confirmed with Sirius Red histopathological studies.

Also noted was loss of microstructure indicated by the presence of irregular and degenerated islets, along with vacuolization and necrosis of β cells accompanied by invasion of inflammatory cells. The sizes as well as the numbers of the pancreatic islets were significantly diminished in DBTC-treated compared with the sham-treated animals (Figure 5B). A few small and shrunken islets were scattered throughout the pancreatic parenchyma, but overall loss of β cells was evident. In addition, pancreatic ducts had become thickened and expanded containing traces of debris and calculi formation (Figure 6A). Extent of pancreatic damage scored (0- normal to 4 most severe) were 3.6 ± 0.4 (severe) in DBTC-treated in contrast to score 0 (normal) for sham-treated animals.

Gall bladder

Gall bladder showed extensive expansion with ductal distension and occasional detected bile stones (Figure 6B).

Hepatitis

Hepatic tissues became enlarged, friable and pale or yellowish in color with a spotted appearance indicating moderately severe hepatitis. Macroscopic hepatic injuries were evidenced with activation of stellate cells, degeneration of hepatocytes, and multifocal and central necrosis (Figure 7A). Additionally, hepatic structure showed fatty degeneration of hepatocytes, and periportal infiltration of inflammatory cells, along with presence of visible ductal dilation. Fibrotic thickening was prominent in the vicinity of portal ducts, and surrounding lobular hepatic parenchyma as confirmed with Sirius Red histopathological studies. The severity of hepatitis was scored 3.7 ± 0.2 (severe) in DBTC-treated animals compared to the score 0

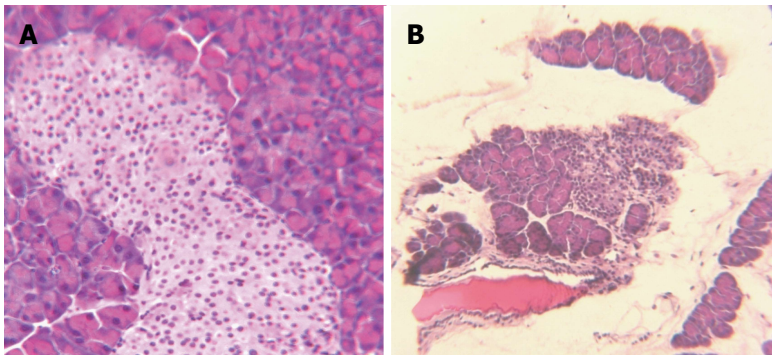

Figure 5 Pancreatic photomicrographs from sham-treated normal control (A) compared to dibutyltin dichloride-treated animal (B). A: Pancreas: Photomicrograph demonstrates sham-treated control pancreas with normal pancreatic microstructure and islet cells. Representative slide from *n* = 8 animals/group; B: Pancreatitis: Photomicrograph illustrates pancreas from a dibutyltin dichloride-treated animal with significant loss of pancreatic structure and islets, acinar atrophy, infiltration of inflammatory cells, fatty deposits and edema (magnification × 40). Representative slide from *n* = 8 animals/group.

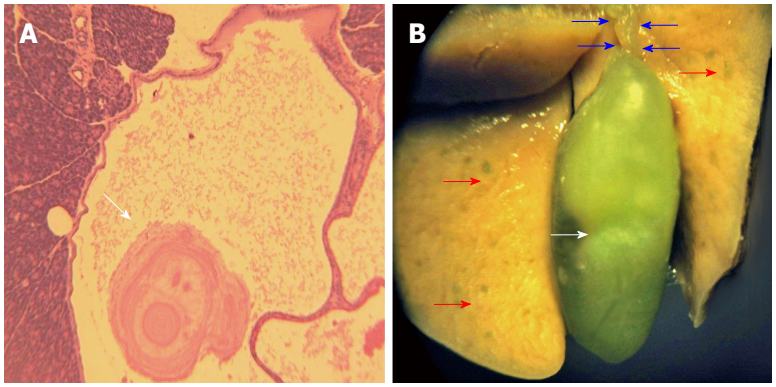

Figure 6 Photomicrograph from pancreatic ductal calculi formation (A), as well as hepatic and gall bladder gross pathological structure (B) in dibutyltin dichloride-treated animals. A: Calculi formation: Photomicrograph illustrates histopathologic structure from a representative pancreas of a dibutyltin dichloride-treated animal to demonstrate ductal distension and calculi formation. Representative slide from *n* = 8 animals/group (magnification × 40); B: Photograph illustrates hepatic gross lesions (red arrows), expanded bile duct (blue arrows) and gall bladder (white arrow) from DBTC-treated animal. Representative from *n* = 8 animals/group.

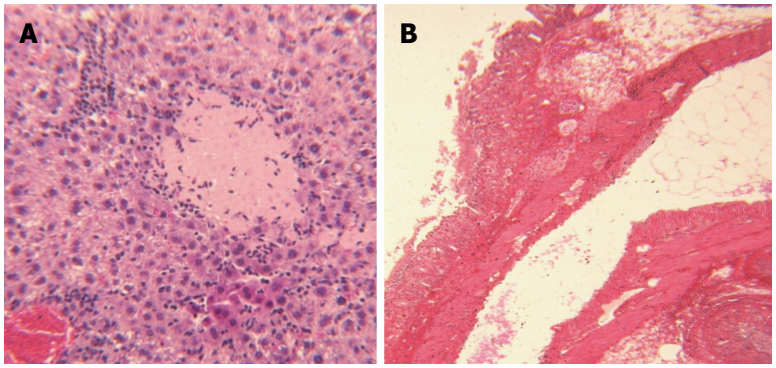

Figure 7 Photomicrograph of hepatitis (A) and colitis (B) from dibutyltin dichloride-treated animals. A: Hepatitis: Photomicrograph illustrates necrosis of hepatocytes, infiltration of inflammatory cells (magnification × 40). Representative slide from *n* = 8 animals/group; B: Colitis: Photomicrograph demonstrates distortion of colonic microstructure, loss of brush boarder epithelial cells, infiltration of inflammatory cells into mucosa and cryptic abscess formation. Representative slide from *n* = 8 animals/group.

(normal) in sham-treated animals.

Colitis

Colonic tissue showed extensive necrosis and loss of intestinal epithelial cells, distortion of cryptic structures, thickening of mucosa due to invasion of inflammatory cells, and some cryptic microabscess formation, presenting advanced colitis (Figure 7B). Colonic lesions and colitis in DBTC-treated animals were scored 3.4 ± 0.3 (moderately severe) compared to 0 (normal) for

the sham-treated animals.

DISCUSSION

Here our findings may provide a new model to better approach investigation of chronic multiorgan inflammatory and visceral pain in murine model and to study possible potential targets in this model for the treatment of chronic hepatobiliary and pancreatitis. Acute pancreatitis is manifested by histopathological transformations, including the presence of inflammatory mediators, acinar atrophy, fat necrosis, intraductal hemorrhage, and stromal proliferation[23]. Chronic pancreatitis is distinguished by recurrent or continuous inflammation of pancreatic progressive atrophy and irreversible fibrosis, with demise of exocrine and endocrine malfunction in severe forms. While, severe and uninhibited abdominal pain is the main aspect of persistent pancreatitis, the mechanism/s by which the pain is induced is poorly explored possibly due to a lack of available appropriate animal model to mimic chronic pancreatitis[24]. In current study the TNFR1/R2 deficient animals displayed significant pain related modifications such as decreases in mechanical threshold after DBTC treatment that persisted through the one month experiment until the end of the study. The significant decreases in mechanical threshold were detected on foot pads and abdomen after the induction of the injury as compared with sham treated animals.

Although, chronic pancreatitis in human is distinguished by irreversible fibrosis, yet pancreatic fibrosis in animal models are mainly reversible[25]. Indeed persistent fibrosis in vital organs results in significant morbidity and mortality worldwide. While, organ fibrogenesis is typically the end product of various non-resolving or repetitive injuries such as chronic infection and radiation exposure; abnormal repair reaction followed by tissue injuries to contribute to the progression of organ fibrosis. Indeed, fibrogenesis is required in natural wound healing process, persistent fibrogenesis in organs can lead to devastating symptoms and organ failure[1,2]. At the cellular level fibrogenesis is remarkably similar progress in different organs and can cause generalized fibrosis in these tissues. Yet, currently there is no appropriate model available to study systemic fibrosis.

Similarly chronic hepatitis and hepatic fibrosis result from excess extracellular matrix produced primarily by hepatic stellate cells. We and other investigators have shown that proinflammatory cytokines (*e.g.,* TNFα) and other inflammatory mediators such as growth factors are regulated by matrix metalloproteinase (MMPs) expressions[20,26]. Further activation of Stellate cells is major event in hepatic fibrosis formation caused by multiple injuries due to chemicals, infectious agents, surgical and/or inflammatory cytokine and chemokines which prompt proliferation and transformation of stellate cells to secret extra cellular matrix. Additionally, the mesenchyme-specific transcription factor forkhead box f1 (Foxf1) in liver is specifically expressed in hepatic stellate cells. Recently, a lipid based liver-specific delivery system also called "dbtc" is reported to be efficient to transfer the Foxf1 siRNA to activated hepatic stellate cells and silence genes expressed in different cell types in liver when used in an acute mouse model of bile duct ligation-induced secondary cholestasis[27].

Pancreatitis models are divided into surgically induced and chemical administration induced hypersecretion of the pancreatic enzymes. The surgical models include ligation and/or cannulation of the biliopancreatic ducts with infusion of variety of solutions, or the formation of closed duodenal loops. Pancreatic fibrosis in bile duct ligated rats is a difficult model to induce and requires increased stimulation. Chemical secretagogues (caerulein or l-arginine) include administration of DBTC to cause a partial blockage of the pancreatic ducts to induce pancreatic disease through enzymic reflux into the gland[28].

Various environmental chemicals have been implicated in the induction of autoimmune responses. Di-n-dibutyltin dichloride is an organotin compound and PVC plastic additive that frequently released to contaminate food and water. As eventually DBTC is degraded in the environment with possible harmful effects on man and animals[29]. Some therapeutic indications of DBTC include at a dose of 10 mg/kg per day for 5 consecutive days effective to eliminate *Trypanosoma brucei* infection in mice[23]. LD50 of DBTC is reported to be 90 mg/kg[30]. Metabolism of DBTC by cytochrome P450 enzymes plays an important role in the induction of biological effects, as DBTC with affinity for mitochondria depresses respiration and elevates serum enzymatic activities resulting in hepatic injuries[31]. Thymus atrophy noted in the current investigation was similar to that reported as a consequence rather than a cause in DBTC-intraperitoneal injected rats[32]. Increased proliferin expression and promotion of morphological thymic transformation reportedly occurring at similar concentrations most probably are DBTC-induced thymus involution. Indeed, this reaction is due to antiproliferative activity of DBTC, as observed by inhibition of thymidine incorporation of thymocytes isolated from DBTC-treated rats[32]. After administration of 4-61 mg/kg iv or 120-240 mg/kg oral DBTC, a dose dependent reversible reduction of thymus weight and number of thymocytes were observed in mice. Iv administration of DBTC highly increased the level of total bilirubin in serum of these animals. But, the level of bilirubin in serum did not correlate with the thymotoxic effects of DBTC in mice[33]. Of interest, toxicity of DBTC in mice is reported after 3 consecutive daily high doses of 50 mg/kg, killing 75% and the survivors developed severe hepatic and bile duct damage. While 3 daily doses of 20 mg/kg caused only mild bile duct and liver lesions[34].

In another study, mice were given DBTC at 8, 15, or 30 mg/kg per day by gavage on days 0-3 or days 4-7 and sacrificed on day 18 of pregnancy. The incidence of embryonic loss increased on days 0-3 at 15 mg or over and, on days 4-7 with 8 mg/kg bw/d and higher. However, no increase in the rate of fetus malformations was observed after the DBTC administration. A decline in the serum progesterone levels was noted in dams given DBTC at 30 mg/kg per day, which might have affected the pregnancy initiation, maintenance, and loss when administered during early pregnancy[35].

Non-alcoholic steatohepatitis (NASH), the most common hepatic disorder, is manifested with inflammation, hepatocyte injury, cell death, fibrosis and multiorganelle failure, leading to cirrhosis[26]. Previously we reported cytokine/chemokine, extracellular matrix accumulation and metalloproteinase upregulation in a dietary deficient NASH model[20]. RT-PCR measurements showed a significant overexpression of inflammatory cytokines [TNFα, transforming growth factor (TGF-β), interleukin (IL-1β), IL-6], suppressor of cytokines signaling1 and genes involved in tissue remodeling and fibrosis (MMPs, collagen-α1) in the hepatic tissues of rats fed methionine-choline deficient diet[20].

Furthermore, using DBTC tail injection rat model we have shown implication of the endothelin cascade gene expression as a major contributing factor in pancreatic pain in both pancreatitis and potential pancreatic cancer[6]. In the present study oral inoculation of DBTC in TNFR1/R2 deficient mice induced a chronic persistent multiorgan hepatobiliary pancreatitis as confirmed by pathological studies including biliary dilation, loss of hepatic and pancreatic architecture and islets, edema in parenchyma, infiltration of inflammatory cells, degeneration, vacuolization and fibrosis, and pancreatic necrosis of acinar cells. Pain related behaviors were increased in animals with pancreatic inflammation including visceral pain-related behavior and secondary cutaneous mechanical hypersensitivity which increased greater than 2-fold. Here lack of TNF receptors appears to accelerate the inflammatory response in multiple organs and contribute to fibrosis in hepatobiliary and pancreatic tissues. A serum proteome profiling analysis in our previous study in TNFR1/R2 deficient mice with pain related behaviors in an arthritis model revealed high levels of serum inflammatory factors. The inflammatory factors included TNFα, which is regulated by the activation of normally T-cell expressed and secreted (RANTES), chemokine (C-X-C motif) ligand 9 [CXCL9 (MIG)], chemokine (C-X-C motif) ligand 10 [CXCL10 (IP-10)], and chemokine (C-C motif) ligand 2 [CCL2 (MCP-1)][10].

Primary sclerosing cholangitis is a complex hepatic disorder, characterized by chronic inflammation of the biliary epithelium, and cholestasis resulting in multifocal bile duct strictures, fibrosis of hepatic parenchyma and biliary tract leading to cirrhosis and malignancy. The etiology of primary sclerosing cholangitis is not fully discovered and no effective therapy is available[5]. Gallstone, one of the most prevalent gastrointestinal complications, is multifactoral, with serious outcomes such as acute gallstone pancreatitis and gallbladder cancer. Gallstone disease is a chronic recurrent hepatobiliary complication which is manifested by creation of gallstones in the hepatic and bile duct, or gallbladder. It is manifested by dysfunctional metabolism of cholesterol, bilirubin and bile acids[7]. Other factors may involve genetic, environmental and steroids. The prevalence of gallstone disease has increased because of sedentary lifestyle and poor diets. Gallstones are known as a common cause of pancreatitis. From 932 patients with acute pancreatitis 40% had gallstones, and 22% alcohol induced[36]. Further, pancreatitis is frequent amongst IBD patients. Gallstones are reported as the most frequent cause of pancreatitis in IBD patients which cause growing diagnostic challenges[37]. Thus, this model may facilitate study of fibrogenesis and/or fibrosis resolution in multiple vital organs leading to development of novel technologies and therapeutic strategies aimed at lessening organ fibrosis.

In conclusions, this is the first report of a chronic inflammatory multiorgan hepatobiliary pancreatitis along with fibrosis and calculi formation model that can be induced reliably with use of oral DBTC administration in TNFR1/R2 deficient mice. Future studies will utilize this model in investigations of anti-fibrotic and analgesic therapeutics.

ACKNOWLEDGMENTS

Dr. Karin N Westlund, PhD, has provided TNFR1/R2 deficient mice and animal facility funded by R01-NS039041 (KNW). Sabrina McIlwrath, PhD, raised the TNFR1/R2 mouse colony. A portion of this paper is selected for presentation in the Metabolic and Genetic Liver Disease Session at the Digestive Disease Week, DDW2016 abstract number 2441753 in San Diego, CA, United States.

COMMENTS

Background

Chronic multiorgan, pancreatitis and hepatobiliary complications manifested with irreversible fibrosis and spontaneous visceral pain in patients. Currently there is no cure or reliable model available for chronic pancreatitis or multiorgan fibrosis other than repeated dosing with chemical caerulein to produce acute flares. An appropriate murine model to mimic the syndrome is desirable. Pancreatic fibrosis in bile duct ligated rats is a difficult model to induce and requires other increased stimulations. The prevalence of gallstone disease has increased because of sedentary lifestyle and poor diets. Dibutyltin dichloride (DBTC) is a biocide, and antifouling agent in the paint and fabric industry. Tail vein injection of DBTC induces unpredictable pancreatitis flares in rats, DBTC injection is tedious, and minor leakage results in tail gangrene and animal loss. TNFα proinflammatory cytokine initiates inflammation through its 2 receptors, TNFR1/R2. We devised a new chronic model of pancreatitis and multiorgan

inflammation in TNFR1/R2 deficient mice using oral DBTC.

Research frontiers

Currently, there is no cure or reliable model available for chronic pancreatitis and multiorgan fibrosis in mice. Persistent pancreatitis manifests with severe abdominal pain, but the mechanism/s by which induced is/are poorly explored possibly due to lack of appropriate models. Three daily doses of 20 mg/kg DBTC caused only mild bile duct and liver lesions, while 3 consecutive daily doses of 50 mg/kg DBTC were toxic and killed 75% of mice. TNFα proinflammatory cytokine initiates inflammation through its 2 receptors, TNFR1/R2. Proteome profiling analysis in our previous study in TNFR1/R2 deficient mice with persistent pain related behaviors in an arthritis model revealed high levels of serum inflammatory cytokines likely responsible for the multiorgan inflammatory response in this model.

Innovations and breakthroughs

This is the first report of a chronic inflammatory hepatobiliary pancreatitis, colitis and stone formation model that can be induced reliably with use of oral DBTC-administration in TNFR1/R2 deficient mice. These findings provide this new murine model to better approach investigation of chronic multiorgan inflammatory and visceral pain in murine mode. In addition, to facilitate study of fibrogenesis and/or fibrosis resolution in multiple vital organs leading to development of novel technologies and therapeutic strategies aimed at lessening organ fibrosis.

Applications

This chronic inflammatory hepatobiliary pancreatitis, colitis/fibrosis and stone formation model can be induced reliably utilizing oral DBTC in TNFR1/R2 deficient mice. The model can be used to investigate chronic multiorgan inflammatory and visceral pain in mice to explore the mechanisms of injury and to study of fibrogenesis and/or fibrosis resolution in multiple vital organs leading to development of novel technologies and therapeutic strategies aimed at lessening organ fibrosis. This study grants ability for further investigation into the use of this model to explore mechanisms of multiorgan injury, the biochemical players and the therapeutic exploration for devastating chronic multiorgn inflammatory and fibrogenesis such as cystic fibrosis and systemic sclerosis as well as cholangitis.

Terminology

TNFR1/R2 deficient mice are transgenic animals lacking tumor necrosis factor receptor 1 and 2 with constant higher proinflammatory TNFα levels compared to wildtype background animals. Multiorgan damage, when 2 or more organs involved in the course of injury. Fibrogenesis is a process usually followed chronic inflammatory to form fibrous structures in and around tissues. Pain related mechanical hypersensitivity, is measured by von Frey microfilament (an accepted standard procedure), demonstrating decreased tolerance to a simple touch manifested with withdraw to protect against induced excess pressure while normal animals tolerate the mechanical touch with no withdrawal response to the fine microfilament touch.

Peer-review

This is an interesting and well-written paper. The author provided that TNFR1/R2 deficient mice treated with DBTC reveal the severe chronic injury of various internal organs which was proved with usage of histological methods.

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P- Reviewer: Khedmat H, Kurzepa J **S- Editor**: Gong ZM
L- Editor: A **E- Editor**: Wang CH

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DOI: 10.3748/wjg.v22.i21.4999

World J Gastroenterol 2016 June 7; 22(21): 4999-5011
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

ORIGINAL ARTICLE

Basic Study

Phosalone-induced inflammation and oxidative stress in the colon: Evaluation and treatment

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Author contributions: Ghasemi-Niri SF performed the majority of experiments and analyzed the data; Baeeri M performed the molecular investigations; Gholami M participated in treatment of animals; Ghasemi-Niri SF, Maqbool F and Abdollahi M designed, coordinated the research and wrote the paper; and Abdollahi M conceived the study.

Supported by Tehran University of Medical Sciences, No. 93-02-45-26666 (in part).

Institutional review board statement: The study was reviewed and approved by the Tehran University of Medical Sciences Institutional Review Board.

Institutional animal care and use committee statement: All procedures involving animals were reviewed and approved by the Institutional Animal Care and Use Committee of the (Tehran University of Medical Sciences) (IACUC protocol: [http://research.tums.ac.ir/informatics/77/Animal%20Research.doc]).

Conflict-of-interest statement: The authors received the partial research funding from Tehran University of Medical Sciences (TUMS). Mohammad Abdollahi is a Professor of Pharmacology and Toxicology at TUMS.

Data sharing statement: Everything about the methods, statistics, and results data are clearly stated in the article with details. No additional data are available.

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Received: February 11, 2016
Peer-review started: February 13, 2016
First decision: March 31, 2016
Revised: April 8, 2016
Accepted: May 4, 2016
Article in press: May 4, 2016
Published online: June 7, 2016

Abstract

AIM: To investigate the side effects of phosalone on intestinal cells and to evaluate benefits of ellagic acid (EA) as a remedy.

METHODS: In order to conduct an *in vivo* study, a rat model was used. The rats were divided into ten groups based on the materials used in the experiment and their dosage. The first group was fed normally. The second group was administered EA through gavage. Next Four groups were given (1/3, 1/5, 1/10, 1/20) LD_{50} phosalone; an organophosphorus compound. The last four groups received (1/3, 1/5, 1/10, 1/20) LD_{50} phosalone and of EA. After one month, the rats were sacrificed and their colon cells were examined to evaluate the level of inflammation, proteins and oxidative stress markers.

RESULTS: The results of this research show that phosalone elevates oxidative stress and changes the level of tumor necrosis factor-a (TNF-α), interlukin-

6β (IL-6β) and nuclear factor (NF)-κB proteins. EA administration reduced phosalone toxicity and changed oxidative stress and inflammatory markers for all phosalone doses. Overall changes in reduction of TNF-α (230.47 ± 16.55 pg/mg protein *vs* 546.43 ± 45.24 pg/mg protein, $P < 0.001$), IL-6β (15.85 ± 1.03 pg/mg protein *vs* 21.55 ± 1.3 pg/mg protein, $P < 0.05$), and NF-κB (32.47 ± 4.85 pg/mg protein *vs* 51.41 ± 0.71 pg/mg protein, $P < 0.05$) manifest that the efficacy of EA is more viable for 1/3 LD_{50} dose of phosalone. Furthermore, EA is effective to counteract the negative outcomes of oxidative stress. When EA was used to treat 1/3 LD_{50} of phosalone's side effects, it improved the level of AChE activity (48.5% ± 6% *vs* 25% ± 7%, $P < 0.05$), TTM (0.391 ± 0.008 mmol/L *vs* 0.249 ± 0.032 mmol/L, $P < 0.05$), FRAP (46.04 ± 5.005 μmol/L *vs* 18.22 ± 1.9 μmol/L, $P < 0.01$) and MPO (0.222 ± 0.019 U/mg protein *vs* 0.387 ± 0.04 U/mg protein, $P < 0.05$).

CONCLUSION: This research highlights that EA is effective to alleviate the side effects of phosalone by reducing the level of oxidative stress and inflammatory proteins.

Key words: Organophosphorus; Phosalone; Ellagic acid; Inflammation; Oxidative stress; Colon

Core tip: This research uses a rat model to evaluate the colon related side effects of phosalone which is a member of the organophosphorus family. After feeding different dosages of phosalone to the rats for one month, the colon tissue of the rats were studied using oxidative stress and pathology tests. Both tests show that the higher doses of phosalone elevate reactive oxygen species (ROS), tumor necrosis factor-α, interlukin-6β and nuclear factor-κB proteins which result in more inflammation. In our study, ellagic acid (EA) which is a strong antioxidant reduced phosalone-induced side effects. The oxidative stress and pathology results concluded that EA helps reducing inflammation and ROS.

Ghasemi-Niri SF, Maqbool F, Baeeri M, Gholami M, Abdollahi M. Phosalone-induced inflammation and oxidative stress in the colon: Evaluation and treatment. *World J Gastroenterol* 2016; 22(21): 4999-5011 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i21/4999.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i21.4999

INTRODUCTION

Pesticides are substances used in agriculture to kill pests and also as a domestic insect killer[1]. Although they are significant in agriculture use but they may also enter human body through inhalation *via* air born particles. Farmers may inhale such chemicals when they use them for pest control[2]. General public is prone to pesticide after eating agricultural products which are not washed properly. Over-usage of pesticides may cause plants to absorb them directly or indirectly through soil. In such case, washing may not completely cleanse the pesticides and their consumers are vulnerable to the resultant side effects[3,4].

Phosalone [O,Odiethyl-S-(6-chloro-2-oxobenzoxazolin-3-yl-methyl)-phosphorodithioate] is a member of the organophosphorus (OP) family, which is used extensively as a pesticide in agriculture and as a domestic insect killer[5]. As compared to Dicoloro Di Three ethane (DDT), phosalone has less severe side effects on human and environment and because of this reason it has replaced DDT for pest control. Regardless of the fact, that phosalone is safer than DDT, but its toxicity has been one of the important research topics in toxicology. The most important known toxicity of phosalone is related to human nervous system. The mechanism of such damage is extremely toxic and phosalone can inhibit neural cholinesterase (ChE) activity, which elevates the level of acetylcholine thus therefore prevents neural signal pathway in the nervous system[6]. Furthermore, like the other members of the OP family, phosalone increases reactive oxygen species (ROS) in the human body tissues thus reduces the level and activity of anti-oxidant enzymes. Higher amount of ROS increases lipid peroxidation (LPO) in the membrane of cells, resulting in membrane damage and disturbance in the cell functional balance[7]. The final repercussions of ROS are faster cell aging and higher chances of DNA and RNA changes, subsequently leading toward cancer and gene mutations[8,9].

The main route through which OP enters the body is mucosa in intestinal cells, where OP can pass through membrane barrier and enter blood. Human cardiovascular system distributes OP to other organs and results in nervous system and ROS related damages[10,11]. Furthermore, the effect of OP on micro flora in intestinal and gastrointestinal enzymes elevate neutrophil infiltration and pro-inflammatory proteins[12,13]. The consequence of such effects is the migration of several immune cells such as neutrophils, monocytes, lymphocytes, macrophages and chemokines then adhesion molecules move toward mucosal tissue. The final outcome of such damage is intestinal inflammation[14,15].

This research elaborates ROS related side effects of phosalone and proposes a material to reduce and potentially eliminate such side effects. The proposed material should be able to offset free-radicals. This research shows that ellagic acid (EA) can be a remarkable candidate to considerably suppress the side effects of phosalone. EA is an important natural occurring substance, which has phenol components[16]. EA is present in numerous fruits and vegetables such

as grapes, nuts, strawberries, black currents, raspberries, green tea, pomegranates, and the stem and bark of Eucalyptus globulus, Eucalyptus maculatu and nuts. The international chemical name of EA is 2,3,7,8-tetrahydroxy-chromeno[5,4,3-cde] chromene-5,10-dione[17].

The biological activities of EA has been investigated in several *in vivo* and *in vitro* studies and have shown that EA has anti-cancer, anti-inflammatory and anti-oxidant properties and in addition it has beneficial therapeutic effect on colon, skin, breast cancer and inflammatory bowel disease (IBD)[18]. EA can also improve mucosa production in goblet cells in colon; reduce pro-inflammatory proteins COX-2 and iNOS over expression and neutrophil infiltration[19]. The anti-oxidant effect of EA stem is clear from the fact, that EA can scavenge free radical, nitrogen reactive species, and ROS, including hydroxyl radicals, peroxyl radicals, NO_2 radicals, and peroxynitrite and therefore EA reduce DNA and cell damages[20]. Additionally, EA can potentially shield DNA and protect it from ROS, free radical and chelation of metal ions attack.

Regarding other effects of EA, some studies have reported that EA can affect cytochrome C in mitochondria which increases BAX/Bcl2, regulates cell division and apoptosis[21]. Also through stimulating the immune system, EA plays a positive role in intercellular complex signaling systems such as mitogen activated protein kinases (MAPKs) and/or the transcription factor nuclear factor κB (NF-κB)[22]. An in-depth study of these effects is presented in this paper.

In our study, we evaluate effect of phosalone on inflammation and oxidative stress with four doses as well as subsequent effect of EA on colon cells.

MATERIALS AND METHODS

Chemicals

Acetylthiocholine iodide, 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) from Merck (Germany), trichloroacetic acid (TCA), Tris base, 1,1,3,3′-tetraethoxypropane (MDA), 2-thiobarbituric acid (TBA), *n*-butanol, 2,4,6-tripyridyl-*s*-triazine (TPTZ), n-butanol, acetic acid, $FeCl_3$-$6H_2O$, benzethonium chloride, 5,5′-Dithiobis(2-nitrobenzoic acid), Trizma® base, EA, o-Dianisidine dihydrochloride, phosphate buffer from Sigma-Aldrich (Germany), n-butanol, hexadecyl tri-methyl ammonium bromide (HETAB), ethylene diamine tetra acetic acid (EDTA), hydrochloric acid (HCL), acetic acid, sodium acetate, hydrogen peroxide (H_2O_2), O-dianisidine hydrochloride, ferric chloride ($FeCl_3$-$6H_2O$), Coomassie reagent, bovine serum albumin (BSA), sodium sulphate (Na_2SO_4), sulphuric acid (H_2SO_4), phosphoric acid (H_3PO_4), potassium dihydrogen phosphate (KH_2PO_4), potassium hydrogen diphosphate (K_2HPO_4), sodium carbonate (Na_2CO_3), cupric sulphate ($CuSO_4$-$5H_2O$) from Merck. Rat-specific tumor necrosis factor-α (TNF-α), interlukin-6 (IL-6) and NF-κB ELISA kits from (Bender MedSystems GmbH, Austria), analytical grade form of phosalone from local pesticide manufacturing companies (Agroxir) and were used in this study.

Experimental animals

In our study, male Wistar rats weighing 180-200 g were selected according to the regulations of the ethics committee of Tehran University of Medical Sciences (TUMS) approved with code number of 93-02-45-26666. Animals were housed separately in standard polypropylene cages with a wire mesh top, kept under standard conditions, including temperature (23 ℃ ± 1 ℃), relative humidity (55% ± 10%) and 12/12 h light/dark cycle, and fed a standard pellet diet and water ad libitum. All ethical themes of studies on animals were considered carefully.

Experiment design

Animals were divided into ten groups based on the materials used in the experiment and their dosage, with six rats in each group. The first group was fed normally. The second group was administered EA (10 mL/kg) through gavage. Next Four groups were given different dosage of phosalone (1/3 LD_{50}: 40 mg/kg, 1/5 LD_{50}: 20 mg/kg, 1/10 LD_{50}: 12 mg/kg and 1/20 LD_{50}: 6 mg/kg), which is a member of organophosphorus family, through gavage. The last four groups received both phosalone (1/3 LD_{50}: 40 mg/kg, 1/5 LD_{50}: 20 mg/kg, 1/10 LD_{50}: 12 mg/kg and 1/20 LD_{50}: 6 mg/kg) and EA (10 mL/kg). After one month, the rats were sacrificed and their colon cells were examined to evaluate the level of oxidative stress factors.

Sample preparation

After 30 d, all rats were anesthetized (40% Ketamine 1000, 25% Xylazine 2%, 0.1 mL/100 g body weight) and after that all of animals were humanly sacrificed and colonic tissues were immediately separated. Isolated segments were rinsed with normal saline and then placed in an ice bath throughout the procedure. Colonic tissue was divided into two pieces. The first piece was weighed and kept in 10 mL of formalin 10%, as a fixator for the purpose of histopathological evaluation. The second piece was weighed and homogenized in 10 volumes of ice cold potassium phosphate buffer (50 mmol, pH = 7.4) and then stored at -20 ℃ for 24 h. The sample was then sonicated and centrifuged for 30 min at 3500 *g*, and the supernatant was transferred to a micro tube. Then sample was kept at -80 ℃ until biomarker analyses.

Determination of lethal dose of phosalone

An lethal dose (LD_{50}) of phosalone is a standard measurement of toxicity that is stated in milligrams (mg) of phosalone per kilogram (kg) of body weight at which 50% of rats are killed. For finding the LD_{50} of phosalone, we performed a study on Wistar rats.

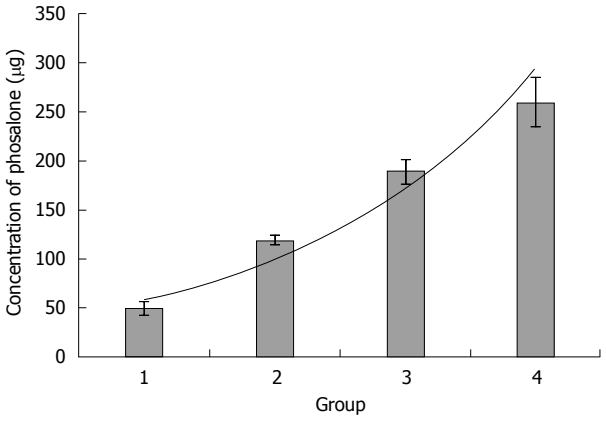


Figure 1 Determination of LD_{50} of phosalone.

We divided five groups of rats and administrated with different doses of phosalone. One of group was control that didn't receive phosalone. But four groups received different doses of phosalone, like 50 mg/kg, 120 mg/kg, 190 mg/kg and 260 mg/kg. After two days we compared all groups and found LD_{50} was between 120 mg/kg to 190 mg/kg. After that we analyzed all data and 120 mg/kg was LD_{50}, used for phosalone in animal model in our study (Figure 1).

Assay of oxidative stress enzymes

AChE activity: AChE activity of erythrocytes was measured according to method of Ellman method using acetylthiocholine iodide as the substrate and 5-5-bis dithionitrobenzoic acid (DTNB). Briefly, 10 μL of sample was added to 3 mL of solution containing 25 mmol/L DTNB in 75 mmol/L phosphate buffer. Then 10 μL of 3 mmol/L acetylcholine iodide was added and absorbance changes were measured at 412 nm in a two-fold rays spectrophotometer[23].

Myeloperoxidase activity assessment

MPO activity was determined by a dianisidine-H_2O_2 method, modified for 96-well plates. Briefly, plasma samples (10 μg protein) were added in triplicate to 0.53 mmol/L o-dianisidine dihydrochloride (Sigma) and 0.15 mmol/L H_2O_2 in 50 mmol/L potassium phosphate buffer (pH 6.0). After incubation for 5 min at room temperature, the reaction was stopped with 30% sodium azide. The absorbance was measured at 460 nm (ε = 11300 $M^{-1}\cdot cm^{-1}$) spectrophotometrically (Shimadzu 160A UV-VIS spectrophotometer). Results were expressed as units of MPO/mg protein, whereby 1 unit of MPO was defined as the amount of enzyme degrading 1 nmol H_2O_2 per min at 25 ℃[24].

LPO measurement

To measure LPO, thiobarbituric acid-reaction substances (TBARS) were assessed in colon tissue. TBA reacts with lipid peroxides in the samples producing a measurable pink color that has absorbance at 532 nm by a double beam spectrophotometer. Concentration of TBARS is recorded as μg[25].

Assay of total thiols

To determine TTM in the control and test groups, 0.6 mL Tris-EDTA buffer (Tris base 0.25 mol/L, ethylene diamine tetra acetic acid 20 mmol/L, pH 8.2) was added to 0.2 mL of supernatant, and after quick vortex mixing, 40 μL 5-5'-dithiobis-2-nitrobenzoic acid (10 mmol/L in pure methanol) was added. The final volume of this mixture was made up to 4.0 mL by an extra addition of pure methanol. After 15 min incubation at room temperature, the samples were centrifuged at 3000 *g* for 10 min and ultimately the absorbance of the supernatant was measured at 412 nm. Data are shown as mmol/L[26].

FRAP assay

Antioxidant power of plasma was evaluated by measuring its ability to reduce of Fe^{3+} tripyridyltriazine (TPTZ) complex (colorless) to Fe^{2+} TPTZ (blue colored) formed by the action of electron donating antioxidants at low pH. The ferric reducing antioxidant power (FRAP) reagent was prepared by mixing 300 mmol/L acetate buffer, 10 mL TPTZ in 40 mmol/L HCl and 20 mmol/L $FeCl_3$ in the proportion of 10:1:1 at 37 ℃. 10 μL of the H_2O diluted sample was then added to 300 mL freshly prepared reagent warmed at 37 ℃. An intense blue color complex was formed when Fe^{3+} TPTZ complex was reduced to Fe^{2+} form. The complex between Fe^{2+} and TPTZ gives a blue color with absorbance at 593 nm. Data are shown as μmol/L[27].

Determination of TNF-α and IL-6β

A human specific ELISA kit (BenderMed System) was used to quantify TNF-α and IL-6 in the supernatant of colon tissue. To assess the amount of TNF-α, the absorbance of sample was measured in 450 nm as the primary wavelength and 620 nm as the reference wavelength by ELIZA reader as described in the kit brochure. TNF-α and IL-6β levels were expressed as pg/mg protein of tissue[28].

Determination of NF-κB

The amount of NF-κB in colon cells extracts was measured by using NF-κB ELISA kits (BenderMed System) according to the manufacturer's instructions. The levels of NF-κB in nuclear extracts were calculated using the standard curve and expressed as pg/mg protein[29].

Total protein assessment

The concentration of protein in the colon homogenate was measured by the Bradford method using BSA as the standard. The absorbance was measured by the spectrophotometer at 595 nm after 5 min. The bovine serum albumin was used as standard[30].

Statistical analysis

At least four independent experiments in repetition were carried away. Data are presented as mean ± SE. One-way ANOVA and Tukey's multi-comparison trials were held out by Stats-Direct 3.0.169 software to determine the statistical differences while the degree of significance had been set at *(P* < 0.05*)*.

RESULTS

Pathology evaluation of the colon damage

As shown in Figure 2, histopathological examination in normal group shows that there was no ulceration, no necrosis, no adhesions, no wall thickening and mucosal/submucosal polymorphonuclear (PMN) leukocyte infiltration. In EA group there was no blood and ulcer in mucosal/submucosal of the colon tissue. In the 1/3 LD_{50} phosalone group, it was observed in some areas infiltration, adhesions, with no any overlying blood and serous adhesion.

The 1/3 LD_{50} phosalone and EA group showed improvement in muscles and mucosa, a reduction inflammation in colon tissue and low lymphocytes infiltration in submucosal layer. The mucosal glands are normal but mild degeneration of mucosal muscle cells and muscle layers is observable. The level of degeneration and inflammation is less than 1/3 LD_{50} phosalone group. Histological examination of 1/5 LD_{50} phosalone and EA group showed improvement in mucosa with the reduction lymphocytes in submucosa region. The mucosal glands are normal but mild degeneration of mucosal muscle cells and muscle layers is observable. The level of degeneration is less than 1/5 LD_{50} phosalone group. In the 1/10 LD_{50} phosalone and 1/20 LD_{50} phosalone with EA groups, the mild degeneration of mucosal muscle cells and muscle layers were observed. In 1/10 LD_{50} phosalone and EA was seen a very mild inflammation due to lymphocytes infiltration between mucosal glands. But in 1/20 LD_{50} phosalone and EA, there was no inflammation in different layers.

AChE activity

After pathological examination, the first step was the evaluation of EA through measurement of AChE activity. AChE activity was reduced in colon cells of groups receiving 1/3 and 1/5 LD_{50} of phosalone in comparison to normal group ($P < 0.01$). In two groups (1/3 and 1/5) LD_{50} phosalone, AChE activity was significantly decreased in comparison to EA group ($P < 0.05$). EA restored the activity of AChE which was suppressed by phosalone. Among different phosalone doses, such AChE activity retrieval was more significant for 1/3 LD_{50} ($P < 0.05$) (Figure 3).

Myeloperoxidase activity

Colonic myeloperoxidase (MPO) activity in 1/3 LD_{50} phosalone group was noticeably higher than that of the normal and EA groups ($P < 0.01$). Data showed a remarkable difference between 1/5 LD_{50} phosalone and EA group ($P < 0.01$). Also, the group of animals which received EA and 1/3 LD_{50} phosalone, showed a reduction of MPO activity (by 26%, $P < 0.05$) in comparison to 1/3 LD_{50} phosalone group (Figure 4).

Oxidative-stress as TBARS

Inflammation in colon referred as over-activity of oxidative stress was found high in 1/3 and 1/5 LD_{50} phosalone groups as compared to normal and EA groups ($P < 0.01$). Colonic lipid peroxidation in 1/10 LD_{50} phosalone group was noticeably higher than that of the normal group ($P < 0.01$). Although, EA decreased oxidative stress in all doses of phosalone, it down-regulated oxidant formation significantly in 1/5 LD_{50} phosalone ($P < 0.05$) (Figure 5).

TTM

An obvious reduction in TTM was observed in 1/3 LD_{50} phosalone group as compared to normal and EA groups ($P < 0.01$). 1/5 and 1/10 LD_{50} phosalone groups significantly decreased TTM in comparison with normal group ($P < 0.05$). EA restored significantly the TTM which was suppressed by 1/3 LD_{50} phosalone (Figure 6).

Anti-oxidant power as FRAP

Less ability in overcoming the oxidative stress in all doses of phosalone groups was reported in contrast to normal and EA groups ($P < 0.001$). FRAP value in 1/3 LD_{50} phosalone was significantly less than EA and 1/3 LD_{50} phosalone group ($P < 0.01$). Amount of FRAP in 1/5 LD_{50} phosalone was markedly lower than its normal content in EA and 1/5 LD_{50} phosalone group ($P < 0.001$). The amount of FRAP increased significantly in EA and 1/10 LD_{50} phosalone group compared to 1/10 LD_{50} phosalone group ($P < 0.001$). A significant increase in FRAP was seen in EA and 1/20 LD_{50} phosalone group when compared to 1/20 LD_{50} phosalone ($P < 0.01$) (Figure 7).

TNF-α level

An obvious rise in TNF-α level was observed in (1/3 and 1/5) LD_{50} phosalone groups as compared to normal group ($P < 0.01$). In (1/3 and 1/5) LD_{50} phosalone groups showed a significant increase in TNF-α level in comparison with EA group ($P < 0.05$). A noticeable improve in TNF-α content was seen in EA and 1/3 LD_{50} phosalone group when compared with 1/3 LD_{50} phosalone group ($P < 0.001$). In EA and 1/5 LD_{50} phosalone group as shown in Figure 8, EA prevented more secretion of TNF-α when compared to 1/5 LD_{50} phosalone group ($P < 0.05$) (Figure 8).

IL-6β level

All doses of phosalone groups showed a notable elevation in IL-6β level in comparison to normal group

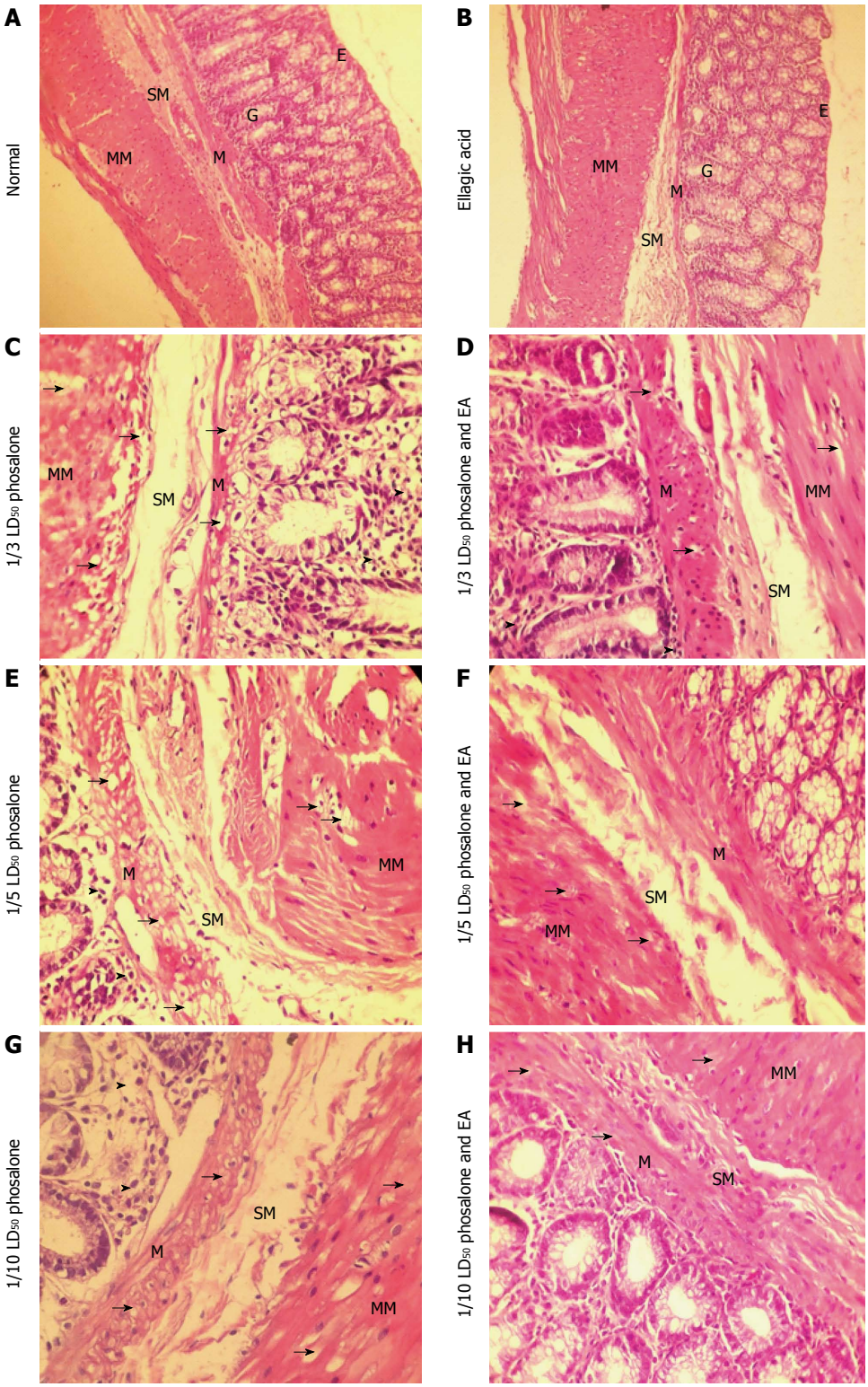
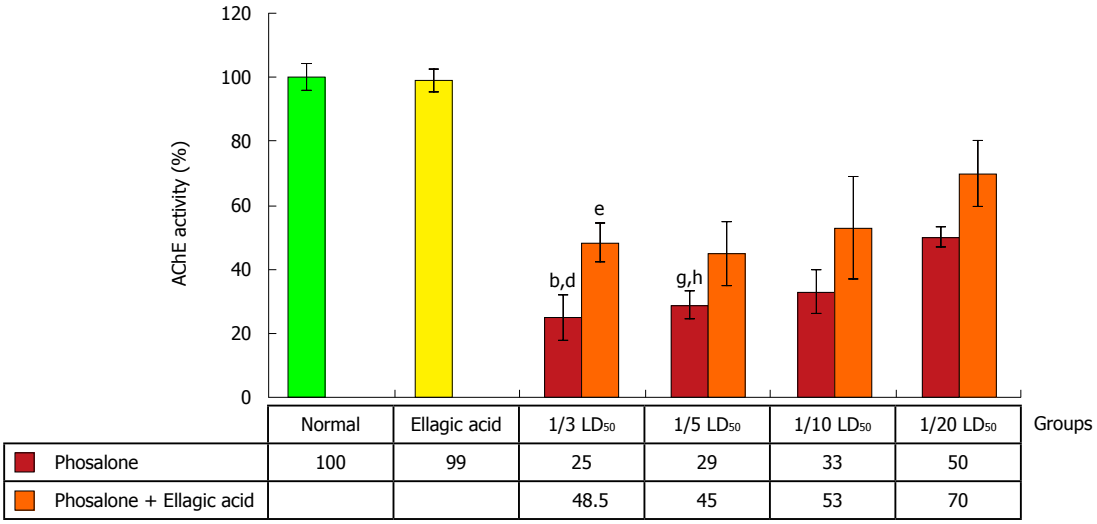


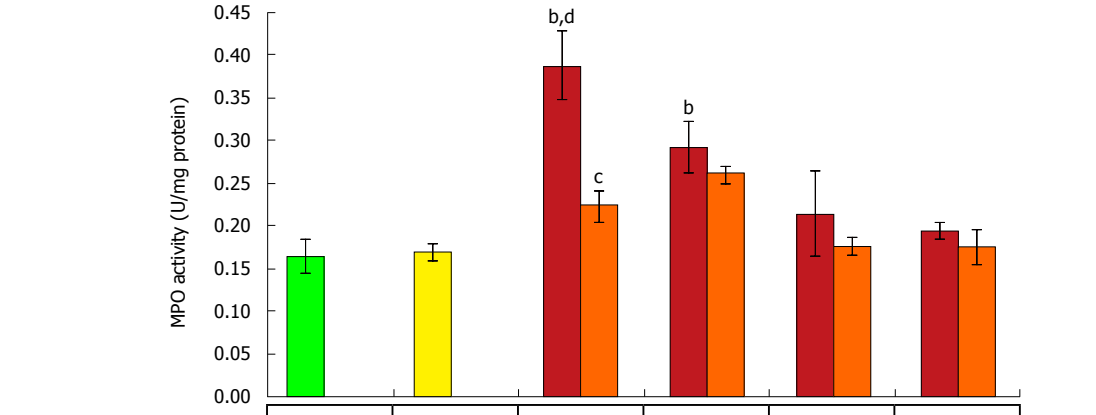
Figure 2 Histological images of colon tissues from normal, ellagic acid and experimental groups. In control group, different parts of the colon tissue are healthy. There are no erosions or ulcers in epithelium. It cannot be seen any necroses and inflammation cells in mucus and mucosal glands in the lamina propria. The mucus thickness, the size of the glands, the muscle layer of mucosal and serous is normal. No degeneration, swelling and goblet cells are observable (A). In ellagic acid (EA) group, the following are normal, the mucosal, the goblet gland cells, epithelium, the mucosa thickness and the size of the glands. There are no ulcers, necrosis hyperplasia and inflammation cells such as neutrophils or lymphocytes. It cannot be observed any degeneration, swelling and goblet cells. The serous is normal without any adherent (B). In 1/3 LD_{50} phosalone group, no necrosis and ulcer are visible in epithelium. The mucosal glands are normal but severe degeneration of mucosal muscle cells and muscle layers is observable. In addition to hyperemia, there is a significant infiltration of mononuclear inflammatory cells such as lymphocytes and plasma cells between the mucosal glands. There is no fibrosis and serous adhesion (C). In 1/3 LD_{50} phosalone group and EA, no necrosis and ulcer are visible in epithelium. The mucosal glands are normal but mild degeneration of mucosal muscle cells and muscle layers is observable. The level of degeneration and inflammation is less than 1/3 LD_{50} phosalone group. There is a very mild inflammation due to lymphocytes infiltration between mucosal glands. There is no serous adhesion (D). In 1/5 LD_{50} phosalone group, no necrosis and ulcer are visible in epithelium. The mucosal glands are normal but relatively severe

degeneration of mucosal muscle cells and muscle layers is observable. In addition to hyperemia, there is a significant infiltration of mononuclear inflammatory cells such as lymphocytes and plasma cells between the mucosal glands. There is no fibrosis and serous adhesion (E). In 1/5 LD_{50} phosalone group and EA, no necrosis and ulcer are visible in epithelium. The mucosal glands are normal but mild degeneration of mucosal muscle cells and muscle layers is observable. The level of degeneration is less than 1/5 LD_{50} phosalone group. There is a very mild inflammation due to lymphocytes infiltration between mucosal glands. There is no serous adhesion (F). In 1/10 LD_{50} phosalone group, no necrosis and ulcer is present in epithelial. The mucosal glands are normal but significant degeneration of muscle cells and mucosal layer is observable. In addition to hyperemia, there is a mild infiltration of mononuclear inflammatory cells such as lymphocytes between the mucosal glands. There is no fibrosis and serous adhesion (G). In 1/10 LD_{50} phosalone group and EA, no necrosis and ulcer are visible in epithelium. The mucosal glands are normal but mild degeneration of mucosal muscle cells and muscle layers is observable. The level of degeneration is less than 1/10 LD_{50} phosalone group. There is a very mild inflammation due to lymphocytes infiltration between mucosal glands. There is no serous adhesion (H). In 1/20 LD_{50} phosalone group, there is no evidence of necroses, ulcer or inflammation in epithelium. Mucosal glands are normal but a mild degeneration is observable in muscle cells in mucosal layer. A mild diapedesis, hyperemia and inflammation in mucosal is visible. There is no fibrosis and serous adhesion (I). In 1/20 LD_{50} phosalone group and EA, no necrosis and ulcer are visible in epithelium. The mucosal glands are normal but mild degeneration of mucosal muscle cells and muscle layers is observable. The level of degeneration is less than 1/20 LD_{50} phosalone group. There is no inflammation in different layers (J). ML: Muscular layer; SM: SubMucosa; M: Mucosa; G: Gland; E: Epithelium.



	Normal	Ellagic acid	1/3 LD_{50}	1/5 LD_{50}	1/10 LD_{50}	1/20 LD_{50}	Groups
Phosalone	100	99	25	29	33	50	
Phosalone + Ellagic acid			48.5	45	53	70	

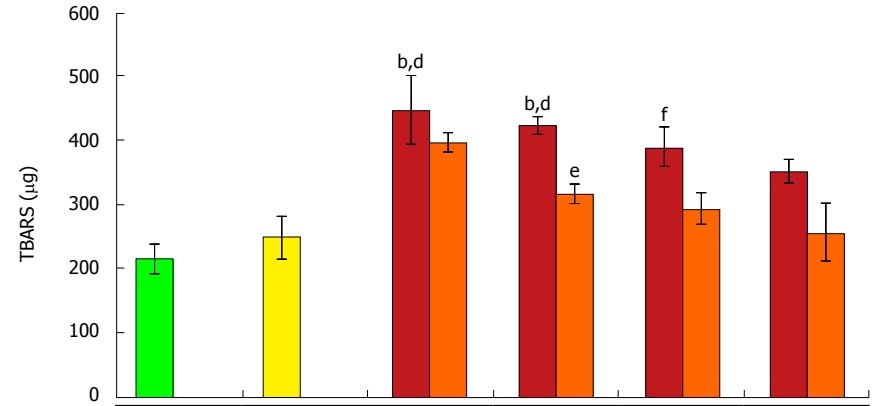
Figure 3 Effect of phosalone and ellagic acid on AChE activity of colon cells. Values are mean ± SE. $^{b}P < 0.001$ *vs* normal group; $^{d}P < 0.001$ *vs* ellagic acid group; Ellagic acid significantly increased of AChE activity in 1/3 dose of phosalone group. $^{e}P < 0.05$ *vs* (1/3 LD_{50} phosalone) group; $^{g}P < 0.05$ *vs* ellagic acid group, $^{h}P < 0.01$ *vs* normal group.



	Normal	Ellagic acid	1/3 LD_{50}	1/5 LD_{50}	1/10 LD_{50}	1/20 LD_{50}	Groups
Phosalone	0.163	0.169	0.387	0.292	0.214	0.194	
Phosalone + Ellagic acid			0.222	0.260	0.175	0.175	

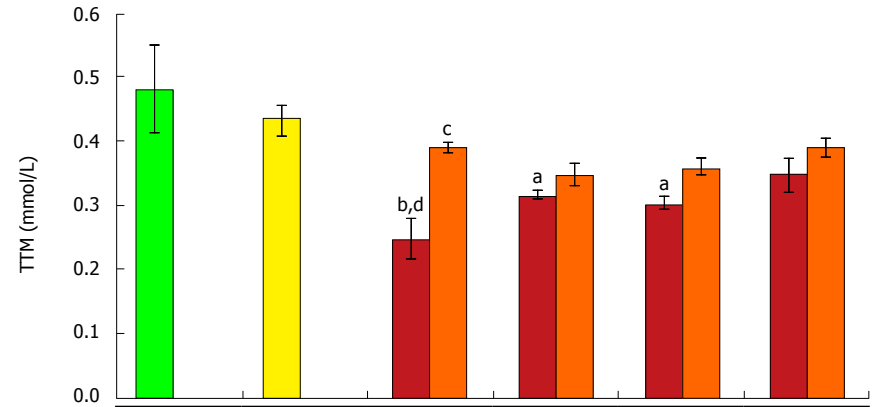
Figure 4 Effect of phosalone and ellagic acid on myeloperoxidase activity of colon cells. Ellagic acid significantly decreased of MPO in 1/3 dose of phosalone group. Values are mean ± SE. $^{b}P < 0.001$ *vs* normal group; $^{c}P < 0.05$ *vs* (1/3 LD_{50} phosalone) group; $^{d}P < 0.01$ *vs* Ellagic acid group. MPO: Myeloperoxidase activity.

($P < 0.001$). The EA and 1/3 dose of phosalone group differed from 1/3 LD_{50} phosalone group remarkably ($P < 0.05$). IL-6β level in 1/3 LD_{50} phosalone group was noticeably higher than that of the EA group ($P < 0.001$). There was significant variation between EA, and EA and 1/5 LD_{50} phosalone groups ($P < 0.01$), while EA and 1/10 LD_{50} phosalone group had a less potency in decreasing IL-6β level when compared to EA group (P



	Normal	Ellagic acid	1/3 LD_{50}	1/5 LD_{50}	1/10 LD_{50}	1/20 LD_{50}	Groups
Phosalone	214.16	247.94	447.46	422.88	390.030	351.88	
Phosalone + Ellagic acid			395.98	317.10	293.516	256.57	

Figure 5 Effect of phosalone and ellagic acid on oxidative-stress as thiobarbituric acid-reaction substances of colon cells. Ellagic acid significantly decreased of thiobarbituric acid-reaction substances in 1/5 dose of phosalone group. Values are mean ± SE. $^{b}P < 0.001$ *vs* normal group; $^{d}P < 0.01$ *vs* Ellagic acid group; $^{e}P < 0.05$ *vs* (1/5 LD_{50} phosalone) group; $^{f}P < 0.01$ *vs* normal group.



	Normal	Ellagic acid	1/3 LD_{50}	1/5 LD_{50}	1/10 LD_{50}	1/20 LD_{50}	Groups
Phosalone	0.482	0.433	0.249	0.317	0.304	0.347	
Phosalone + Ellagic acid			0.391	0.349	0.360	0.391	

Figure 6 Effect of phosalone and ellagic acid on total thiol molecules activity of colon cells. Ellagic acid significantly increased of total thiol molecules in 1/3 dose of phosalone group. Values are mean ± SE. $^{a}P < 0.05$ *vs* normal group; $^{b}P < 0.01$ *vs* normal group; $^{c}P < 0.05$ *vs* (1/3 LD_{50} phosalone) group; $^{d}P < 0.01$ *vs* Ellagic acid group.

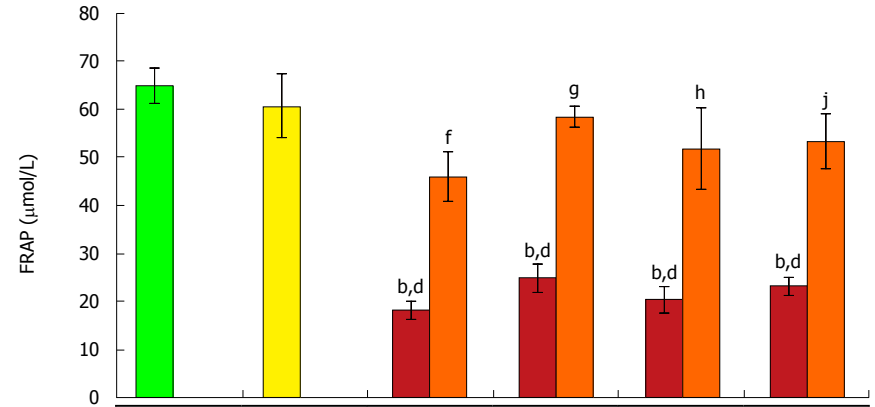
< 0.05) (Figure 9).

NF-κB release

As seen in Figure 10, NF-κB production was significantly elevated in the (1/3 and 1/5) LD_{50} phosalone groups when compared with normal group ($P < 0.001$). A significant increase in NF-κB was seen in (1/10 and 1/20) LD_{50} phosalone groups when compared with normal ($P < 0.05$). The EA and 1/3 LD_{50} phosalone group showed more reduction in NF-κB when compared with 1/3 LD_{50} phosalone ($P < 0.05$). The (1/3, 1/5 and 1/10) LD_{50} phosalone groups which were treated with EA showed an apparent increase in NF-κB level when compared with EA group *(*$P < 0.01$*)*.

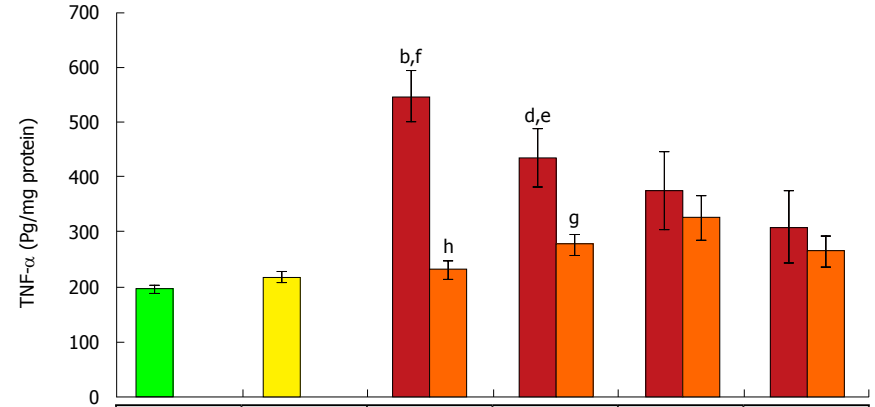
DISCUSSION

In our study we succeeded to achieve our main hypothesis: to find phosalone toxicity in colonic tissues of rats as well as protective effects of EA, during subchronic exposure. Phosalone is type of OP pesticide that could affect different organs in daily and produce various toxicities[31]. As a result of phosalone exposure in rats, increase in oxidative stress and inflammatory markers were observed. In our experiment, EA was used to reduce colon injury induced by phosalone as a protective agent, which showed substantial decrease in oxidative stress and inflammatory markers. EA that is kind of polyphenol derived from different plants



	Normal	Ellagic acid	1/3 LD_{50}	1/5 LD_{50}	1/10 LD_{50}	1/20 LD_{50}	Groups
Phosalone	64.79	60.65	18.22	24.700	20.311	23.25	
Phosalone + Ellagic acid			46.04	58.375	51.790	53.37	

Figure 7 Effect of phosalone and ellagic acid on anti-oxidant power as (ferric reducing antioxidant power) of colon cells. Ellagic acid significantly decreased of ferric reducing antioxidant power in all doses of phosalone groups. Values are mean ± SE. $^{b}P < 0.001$, *vs* normal group; $^{d}P < 0.001$ *vs* ellagic acid group; $^{f}P < 0.01$ *vs* (1/3 LD_{50} phosalone) group; $^{g}P < 0.001$ *vs* 1/5 LD_{50} phosalone; $^{h}P < 0.001$ *vs* 1/10 LD_{50} phosalone; $^{j}P < 0.01$ *vs* 1/20 LD_{50} phosalone.

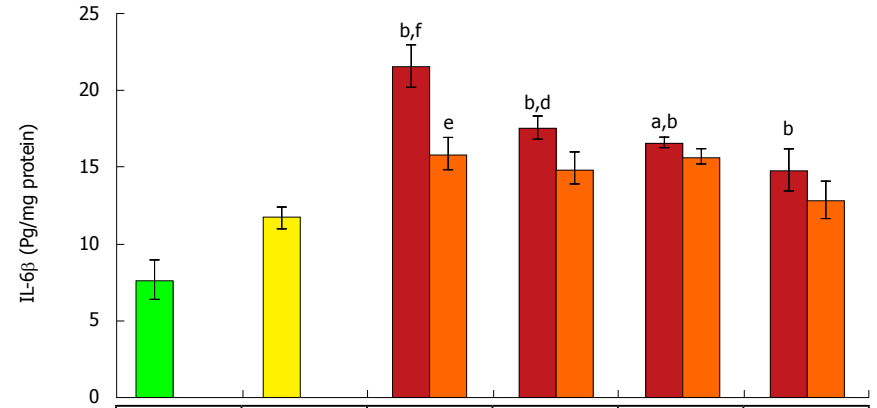


	Normal	Ellagic acid	1/3 LD_{50}	1/5 LD_{50}	1/10 LD_{50}	1/20 LD_{50}	Groups
Phosalone	196	217.9	546.430	433.870	375.125	309.500	
Phosalone + Ellagic acid			230.475	275.355	324.760	264.512	

Figure 8 Effect of phosalone and ellagic acid on tumor necrosis factor-α of colon cells. Ellagic acid significantly decreased of tumor necrosis factor-α in 1/3 and 1/5 doses of phosalone groups. Values are mean ± SE. $^{b}P < 0.001$ *vs* normal group; $^{d}P < 0.01$ *vs* normal group; $^{e}P < 0.05$ *vs* ellagic acid group; $^{f}P < 0.01$ *vs* ellagic acid group; $^{g}P < 0.05$ *vs* (1/5 LD_{50} phosalone) group; $^{h}P < 0.001$ *vs* (1/3 LD_{50} phosalone) group.

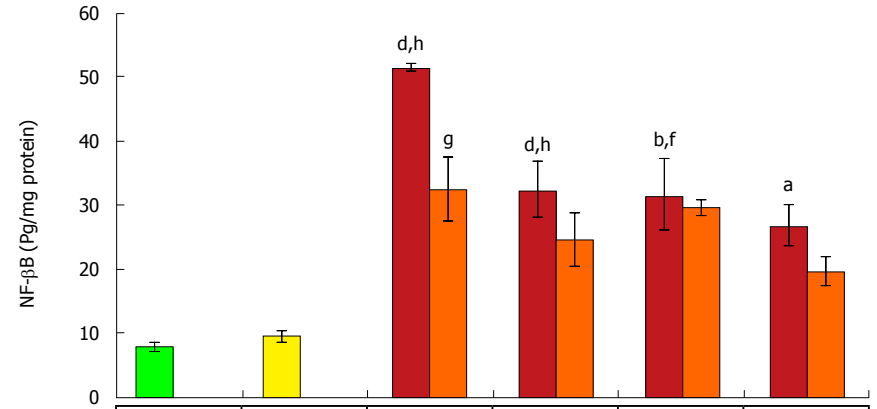
or fruits has already been reported to have sort of protective effects in different diseases[32,33]. As indicated in the present study, AChE activity was reduced with pronounced effect in colon cells of groups receiving (1/3 and 1/5) LD_{50} phosalone in comparison to both normal group and EA group. In previous studies, the same inhibition of AChE was observed during behavioral studies in phosalone-treated rats brain cells[34,35]. AChE inhibition is among best indicator of toxicity induced by any xenobiotic or chemical that initiates other signaling pathways. Despite of little effect in other groups, EA considerably reversed the activity of AChE, suppressed by phosalone in group receiving 1/3 LD_{50} phosalone. It has been already published that, by exposure of OPs elevated level of ACh *via* ChE inhibition could interfere with cholinergic receptors within hypothalamus and potentiate release of adrenocorticotropic hormone (ACTH)[36]. The present study proves that phosalone inhibits AChE activity that is associated with colon inflammation and EA could reverse its effect.

Increased production and decreased ability of ROS and antioxidant defense mechanism respectively can damage various signaling pathways, as well as cell constituents, including DNA, lipids and proteins. Induction of such oxidative impairment *via* redox signaling mechanisms can cause different human diseases[37]. In our experiment, biochemical assays showed that phosalone elevated oxidative stress *via* elevation of MPO activity and TBARS concentration, whereas in groups with combined EA administration; reduction in MPO activity and TBARS concentration has been observed. Irrespective of our study on phosalone,



	Normal	Ellagic acid	1/3 LD_{50}	1/5 LD_{50}	1/10 LD_{50}	1/20 LD_{50}	Groups
Phosalone	7.66	11.72	21.55	17.54	16.60	14.83	
Phosalone + Ellagic acid			15.85	14.91	15.67	12.86	

Figure 9 Effect of phosalone and ellagic acid on interlukin-6β of colon cells. Ellagic acid significantly decreased of tumor necrosis factor-α in 1/3 dose of phosalone group. Values are mean ± SE. $^{a}P < 0.05$ *vs* ellagic acid group; $^{b}P < 0.001$ *vs* normal group; $^{d}P < 0.01$ *vs* ellagic acid group; $^{e}P < 0.05$ *vs* (1/3 LD_{50} phosalone) group; $^{f}P < 0.001$ *vs* ellagic acid group.



	Normal	Ellagic acid	1/3 LD_{50}	1/5 LD_{50}	1/10 LD_{50}	1/20 LD_{50}	Groups
Phosalone	7.78	9.43	51.411	32.36	31.68	26.87	
Phosalone + Ellagic acid			32.470	24.60	29.56	19.65	

Figure 10 Effect of phosalone and ellagic acid on nuclear factor-κB of colon cells. Ellagic acid significantly decreased of nuclear factor-κB in 1/3 dose of phosalone group. Values are mean ± SE. $^{a}P < 0.05$ *vs* normal group; $^{b}P < 0.01$ *vs* normal group; $^{d}P < 0.001$ *vs* normal group; $^{f}P < 0.01$ *vs* ellagic acid group; $^{h}P < 0.001$ *vs* ellagic acid group; Significantly different from at $^{g}P < 0.05$ *vs* (1/3 LD_{50} phosalone) group.

number of previous studies and literature demonstrate a close relation between exposure of pesticides and occurrence of various health problems *via* induction of oxidative stress[38,39]. A study conducted on humans *via in vitro* setup concluded that: oxidative stress and ROS were increased due to OPs exposure[40]. On other hand a pronounced effect of EA against free radical formation can be seen in groups receiving (1/3 and 1/5) LD_{50} phosalone. However, it has been reported that both MPO and TBARS are indicators of oxidative stress and colon inflammation[41,42]. In addition to this, in our study, body's antioxidant defense mechanism was targeted by phosalone, which caused reduction in TTM and FRAP concentration as compared to normal and EA groups. A significant effect of EA as antioxidant has been observed in all groups receiving both EA and phosalone in different doses. Protective and beneficial effects of EA in oxidative stress has been previously evidenced in many studies[43,44]. It can be derived from current biochemical tests that colon tissues of rats are prone to OPs like Phosalone and thus EA can better treat colon tissue damage *via* different mechanisms, while further studies can be conducted for treatment of IBD (inflammatory bowel disease). Oxidative stress and its balance is the most significant feature of normal physiology, in case of high toxicity it can initiate many signaling pathways that can lead to cell death. Our study shows that, EA play its role as protective agent in oxidative impairment, against free radical production and colitis due to phosalone exposure. So in colon inflammation induced by pesticides, EA can be used as anti-inflammatory, anticancer and antioxidant.

Furthermore we evaluated the effect of phosalone on different inflammatory markers along with protective effect of EA. Our concept regarding toxic mechanisms in colon inflammation is growing and general overview is that T cells secrete IL-2, IL-1B and Interferon-c (IFN-c) that can excite macrophages to release extra TNF-α and IFN-c, ROS and other inflammatory mediators. Occurrence of TNF-α, IL-1B, ROS and some antigens target other signaling pathways which ultimately result in synthesis of cytokines[45]. TNF-α employs its action through elevating the synthesis of inflammatory mediators like IL-1 and IL-6[46]. Our recent study shows that phosalone increase TNF-α and EA well treat such condition in colon inflammation, which require further research regarding IBD. Phosalone caused increase in TNF-α level in all groups with significant change in group 1/3 LD_{50} phosalone and 1/5 LD_{50} phosalone as compared to normal and EA groups. EA showed prominent effect as protective agent to reduce TNF-α level in all groups with significant change in group 1/3 LD_{50} phosalone and 1/5 LD_{50} phosalone as compared to others.

In case of biomarker IL-6β, our study showed consistent finding and phosalone caused increase in IL-6β in all groups significantly as compared to normal and EA groups, whereas EA reversed its effect in all groups with significant change in group 1/3 LD_{50} phosalone as compared to EA group. It is common belief that NF-κB shows its significant function in expression of various inflammatory mediators. NF-κB controls transcriptional activity involved in inflammatory and immune process *via* binding to specific DNA sequences in inflammatory genes[47]. In the same pattern, NF-κB was increased in all groups receiving phosalone with significant change. Contrary to this EA reduced its concentration in almost all groups with significant effect of group receiving 1/3 LD_{50} phosalone. In parallel to our current study same effects of EA as anti-inflammatory agent has been observed in previous experiment[48]. It is clear from our results that how EA and phosalone target different biochemical pathways of toxicity. These distinct properties make NF-κB a promising target in novel treatment plans. There are new techniques that directly target NF-κB in inflammatory conditions including antioxidants, antisense DNA targeting, and proteasome inhibitors. Parallel to our study, a previous study also demonstrated that antioxidant effect may also give boost to anti-inflammatory actions[49]. However, EA's mechanism of actions to offset phosalone toxicity can be further studied regarding signaling pathways and gene expressions. Our research outcomes can give new directions, regarding novel treatment plans of colitis as well as awareness of phosalone toxicity in colon tissues.

Our data correlate well with the other studies and demonstrate that phosalone is among one of causative agents to induce colon inflammation and EA is an ideal antioxidant and anti-inflammatory compound in rat modeling studies which has extraordinary effects on oxidant and inflammation systems. Anyhow, additional investigation for *in vivo* and human studies is required. It may indicate a new way toward the development of antioxidant therapy for colon inflammation.

COMMENTS

Background

Pesticides are chemical agents which are used to kill agricultural and domestic insects. Some of the pesticides are based on Organophosphorus (OP) compounds which are also harmful for human and can lead to early aging and cancer. Understanding the mechanism of action of OPs in human body is of prime importance in recent years. Such understanding will lead to finding the means to counteract the side effects resulted from OP exposure. Phosalone is an OP compound used in this study.

Research frontiers

Prior researches have shown that OP exposure causes inflammation and oxidative stress in the body. The previous and on-going research efforts report serious damages to DNA, RNA and cell cycle due to OP agents.

Innovations and breakthroughs

This research confirms the side effects of OP in colon cells in a rat model. Such side effects are the elevated level of inflammation and oxidative stress. The research results shows that among four dosages of phosalone, highest dosage leads to the most significant and serious level of inflammation and oxidative stress. To alleviate such deteriorative side effects, this research proposes utilizing ellagic acid (EA) which is a strong antioxidant. When rats were given EA along with phosalone, the level of inflammation and oxidative stress reduced significantly for the highest dose of phosalone.

Applications

The results of this research can initiate appropriate warnings and precautions to all individuals including farmers who are exposed excessively to OP compounds. Such individuals can be directed to include EA in their diet through taking EA tablets or eating the fruits and vegetable which are rich source of antioxidants and EA like strawberries, grapes and green tea.

Terminology

Reactive oxygen species (ROS) is a physiological process which happens when the body defense system gets triggered due to inflammation and oxidative stress. ROS leads to variety damages to DNA, RNA and cells.

Peer-review

This study is very significant and interesting. The authors have done standard measurements of toxicity, and demonstrated EA can be used to reduce oxidative stress and regulate the level of inflammatory proteins. EA maybe a good candidate which can help treat and alleviate the side effects induced by OP compounds.

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P- Reviewer: Fujino Y, Liu B **S- Editor**: Ma YJ **L- Editor**: A **E- Editor**: Ma S

World Journal of Gastroenterology

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Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.3748/wjg.v22.i21.5012

World J Gastroenterol 2016 June 7; 22(21): 5012-5022
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

ORIGINAL ARTICLE

Basic Study

CdSe/ZnS quantum dots induce photodynamic effects and cytotoxicity in pancreatic cancer cells

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Author contributions: He SJ and Cao J contributed equally to this study; He SJ, Cao J and Xu LM performed the majority of the experiments; He SJ drafted the manuscript; Cao J and Xu LM designed the study and supervised its implementation; Li YS, Yang JC, Zhou M, Li MM and Qu CY participated in the experiments; Zhang Y, Shen F and Chen Y analyzed the data; all authors made critical revisions and approved the final version to be published.

Supported by the National Natural Science Foundation of China, No. 81472844 and No. 81301826; and Shanghai Municipal Education Commission, No. 14ZZ114.

Institutional review board statement: The study was reviewed and approved by the Xinhua Hospital Affiliated to Shanghai Jiao Tong University School of Medicine Institutional Review Board.

Conflict-of-interest statement: The authors declare that there is no conflict of interest related to this study. The abstract of this paper was presented at AGA Digestive Disease Week as a poster with interim findings, and was published in "Poster Abstracts" in Gastroenterology: http://www.sciencedirect.com/science/article/pii/S0016508515332121.

Data sharing statement: Technical appendix, statistical code, and dataset are available from the corresponding author at leiming.xu@aliyun.com. Participants gave informed consent for data sharing.

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Received: January 18, 2016
Peer-review started: January 19, 2016
First decision: February 18, 2016
Revised: March 4, 2016
Accepted: March 18, 2016
Article in press: March 18, 2016
Published online: June 7, 2016

Abstract

AIM: To investigate the photodynamic effect of CdSe/ZnS quantum dots (QDs) on pancreatic cancer cells and elucidate the probable mechanisms.

METHODS: The pancreatic cancer cell line SW1990 was treated with different concentrations of CdSe/ZnS QDs (0, 0.5, 1.0, 1.5, 2.0, 2.5 μmol/L), with or without illumination. The viability of SW1990 cells was tested using the Cell Counting Kit-8 (CCK-8) assay. The ultrastructural changes of SW1990 cells were observed by transmission electron microscopy. Apoptosis was detected by nuclear staining and flow cytometry (FCM). Reactive oxygen species (ROS) were measured

by dichlorofluorescein diacetate *via* fluorescence microscopy. Expression of Bax, Bcl-2 and caspase-3 was measured by real-time polymerase chain reaction (PCR) and protein immunoblotting 24 h after SW1990 cells were treated with CdSe/ZnS QDs and illuminated.

RESULTS: The CCK-8 assay results showed that both CdSe/ZnS QDs with and without illumination suppressed SW1990 cell proliferation. Cell viability was significantly lower when illuminated or with a longer incubation time and a higher light dose. CdSe/ZnS QDs with illumination caused ultrastructural changes in SW1990 cells, such as organelle degeneration and chromatin condensation and aggregation at the periphery of the nucleus. Fluorescence microscopy and FCM showed that CdSe/ZnS QDs (1.5 μmol/L) with illumination increased SW1990 cell apoptosis (53.2%) and ROS generation compared with no illumination. Real-time PCR showed that expression of Bax and caspase-3 was upregulated and Bcl-2 was downregulated. Immunoblotting results were consistent with real-time PCR results. Inhibition of ROS and apoptosis both attenuated QD-photodynamic-therapy-induced cell death.

CONCLUSION: CdSe/ZnS QDs can be used as a photosensitizer to inhibit SW1990 cell proliferation through ROS generation and apoptotic protein expression regulation.

Key words: Quantum dots; Pancreatic cancer; Apoptosis; Photodynamic therapy; Reactive oxygen species

Core tip: This study showed that quantum dots (QDs) may be a potential photosensitizer for photodynamic therapy (PDT) to treat pancreatic cancer by inhibiting SW1990 cell proliferation and inducing apoptosis through reactive oxygen species (ROS) generation. QD-PDT may induce apoptosis through ROS-, caspase-3-mediated apoptotic pathways, with upregulation of apoptosis signaling molecules such as Bax and downregulation of Bcl-2. These findings provide a new application for PDT in pancreatic cancer. However, more preclinical and clinical trials should be undertaken before further clinical application.

He SJ, Cao J, Li YS, Yang JC, Zhou M, Qu CY, Zhang Y, Shen F, Chen Y, Li MM, Xu LM. CdSe/ZnS quantum dots induce photodynamic effects and cytotoxicity in pancreatic cancer cells. *World J Gastroenterol* 2016; 22(21): 5012-5022 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i21/5012.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i21.5012

INTRODUCTION

Pancreatic cancer is a malignant neoplasm with a very poor prognosis. The 5-year survival is < 5% and medium survival is about 6 mo[1]. Surgical resection is the first-choice treatment for pancreatic cancer, however, 80% of patients may already have locally advanced or metastatic cancer when diagnosed, and only 10%-15% are eligible for surgery[2]. The majority of pancreatic cancer patients have to undergo radiotherapy or chemotherapy, although the survival rates of these nonsurgical patients are similar. Thus, there is an urgent need to identify a novel effective therapy.

Photodynamic therapy (PDT) is an innovative method that utilizes a photosensitizing agent or photosensitizer (PS) followed by light exposure to treat various diseases. Reactive oxygen species (ROS) are generated when PSs are activated by illumination and subsequently destroy cancer cells[3]. In PDT, only cells in contact with the PS, light and oxygen are affected, thus PDT is more selective than conventional chemotherapy and radiotherapy[4]. Most PSs are based on a tetrapyrrole structure. The first PS used clinically for cancer therapy was hematoporphyrin derivative, a water-soluble mixture of porphyrins. As hematoporphyrin derivative is purified from porfimer sodium, it has some disadvantages, such as instability in aqueous solution, long-lasting skin photosensitivity, and weak absorption at the therapeutic wavelength of 630 nm[5]. 5-aminolevulinic acid is a second-generation PS. It is a biosynthetic precursor of protoporphyrin IX that needs to be converted to protoporphyrin as an active PS[6]. However, its skin photosensitivity is still an unresolved problem[7]. Therefore, it is necessary to develop new PSs to confer survival benefits with fewer side effects.

Quantum dots (QDs) are colloidal semiconductors and mainly composed of group Ⅱ-Ⅵ or group Ⅲ-Ⅴ elements[8]. QDs are of interest to many researchers due to their unique optical properties. QDs possess several characteristics such as large absorption spectra, narrow and symmetric emission bands, and a high molar extinction coefficient, which make them superior to conventional PSs in PDT[9,10]. Recently, many studies have shown the potential applications of QDs for PDT. With illumination, the QD conduction-band electron can be transferred to surrounding O_2 and produce ROS, thus making QDs a potential PS for PDT[11,12].

In this study, we prepared water-dispersible CdSe/ZnS QDs with an extensive absorption in the UV-visible region and a strong emission peaking at 560 nm. We investigated the photodynamic effects of CdSe/ZnS QDs on pancreatic cancer cells, and analyzed the possible molecular mechanism involved in this procedure.

MATERIALS AND METHODS

QD nanocrystal characterization

Nanocrystals with a CdSe core and ZnS shell were synthesized by Professor Zhang *et al* at the Department of Materials Science and Engineering, Shanghai

University. Trioctylphosphine oxide, CdO and tetradecylphosphonic acid were heated to 180 ℃ under argon, exsiccated and exhausted under a vacuum. When the reaction temperature reached 330 ℃, selenium precursor solution was added to trioctylphosphine and mixed together until the temperature decreased to 240 ℃. ZnS stock solution was added along with dimethyl zinc solution and vigorously stirred until the molar ratio of Cd/Se: Zn/S reached 1:4. The mixture was cooled to room temperature and settled with anhydrous methanol, centrifuged (4000 rpm) and washed three times with anhydrous methanol to remove residua such as trioctylphosphine oxide and unreacted reagents. The precipitate was suspended in phosphate-buffered saline (PBS). The morphology of QDs was observed using a Morgagni 268(D) transmission electron microscope (FEI, Hillsboro, OR, United States). The UV-Vis spectra of the QDs suspensions were scanned within the wavelength range of 200-800 nm at 22 ℃ and automatically corrected for the suspension, using an Avantes UV-Vis spectrophotometer (Apeldoorn, The Netherlands).

Cell culture

The human pancreatic cancer cell line SW1990 was purchased from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). The SW1990 cells were cultured in RPMI 1640 media with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin, in humidified air containing 5% CO_2 at 37 ℃. Cells in the exponential growth phase were used in the following experiments.

Cytotoxicity assays

The cytotoxicity induced by QDs and PDT was determined using the Cell Counting Kit-8 (CCK-8) assay. The cells were seeded on a 96-well plate at 8×10^3 cells/well and incubated overnight before QDs were added. The cells were divided into two groups (A with illumination and B without illumination). The cells in each group were treated with different concentrations of QDs (0, 0.5, 1.0, 1.5, 2.0, 2.5 μmol/L) for 1 h. After incubation, all cells were washed with PBS to remove excess QDs and fresh media were added. Cells in Group A were irradiated using ZF-20D Ultraviolet Analyzing Equipment at a wavelength of 365 nm and power of 19 mW cm^{-2}, and then incubated in RPMI 1640 medium with 10% FBS for a further 24 h at 37 ℃ in a humidified 5% CO_2 atmosphere. However, the medium in Group B was removed and replaced with RPMI 1640 medium with 10% FBS, and then the cells were incubated in humidified air containing 5% CO_2 at 37 ℃ for a further 24 h. After 24 h incubation, CCK-8 dye (Dojindo Laboratories, Kamimashiki-gun, Kumamoto, Japan) was added to each well, and the 96-well plates were put into a constant temperature incubator (37 ℃) for 1 h. The absorbance of the solution was measured at 450 nm using an ELISA reader (Thermo Fisher Scientific, MA, United States). Cell viability was calculated as a percentage of the treated samples relative to untreated controls.

Subcellular damage of QDs using transmission electron microscopy

Transmission electron microscopy (TEM) was used to investigate the intracellular localization and subcellular structural targets of QDs in SW1990 cells. The cells were seeded on six-well plates at 4×10^5 cells/well and cultured overnight. Twenty-four hours later, after treatment [A: normal SW1990 cells; B: CdSe/ZnS QDs (1.5 μmol/L, 3 h) and illumination; C: CdSe/ZnS QDs (2 μmol/L, 3 h) and illumination (20 J/cm^2)], the cells were collected and washed three times with cold PBS, pelleted using centrifugation (1000 rpm), and fixed in 2.5% glutaraldehyde for 2 h. Cell pellets were washed in PBS, postfixed with 1% osmium tetroxide, and dehydrated with an ascending series of alcohols. The specimens were cut into ultrathin sections (50-70 nm), placed onto copper grids, and stained with uranyl acetate and lead citrate for ultrastructural analysis using a JEM-1011EX transmission electron microscope (Jeol, Tokyo, Japan).

Apoptosis by flow cytometry

The Annexin V-FITC Apoptosis Detection Kit (BD Pharmingen, San Jose, CA, United States) was used to detect QD-induced apoptosis of SW1990 cells. Cells (2×10^5) were seeded in six-well plates and allowed to adhere overnight. The cells were treated [A: normal SW1990 cells; B: SW1990 cells with illumination (20 J/cm^2); C: SW1990 cells treated with CdSe/ZnS QDs (1.5 μmol/L, 3 h); D: SW1990 cells treated with CdSe/ZnS QDs (1.5 μmol/L, 3 h) with illumination (20 J/cm^2)], collected and washed twice with cold PBS. The cell pellets were resuspended in binding buffer, and incubated with staining solution [annexin V/propidium iodide (PI) = 1:1] in the dark for 15 min at room temperature. Fluorescence-activated cell sorting (FACS) analysis was performed using a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, United States).

Measurement of ROS generation

SW1990 cells were seeded on six-well plates at 4×10^5 cells/well and exposed to QDs as for flow cytometry (FCM) after the cells adhered. After 24 h, the cells were rinsed with cold PBS and stained with 2′,7′-dichlorofluorescin diacetate (H_2DCFDA; Sigma-Aldrich, St. Louis, MO, United States) diluted in serum-free medium. After incubation for 30 min at 37 ℃ in the dark, the cells were washed with serum-free medium three times and resuspended in cold PBS. The DCF fluorescence was observed by a fluorescence microscope (BD Biosciences) and the fluorescence intensity was measured by FCM (BD Biosciences, San Jose, CA, United States). To investigate the role of

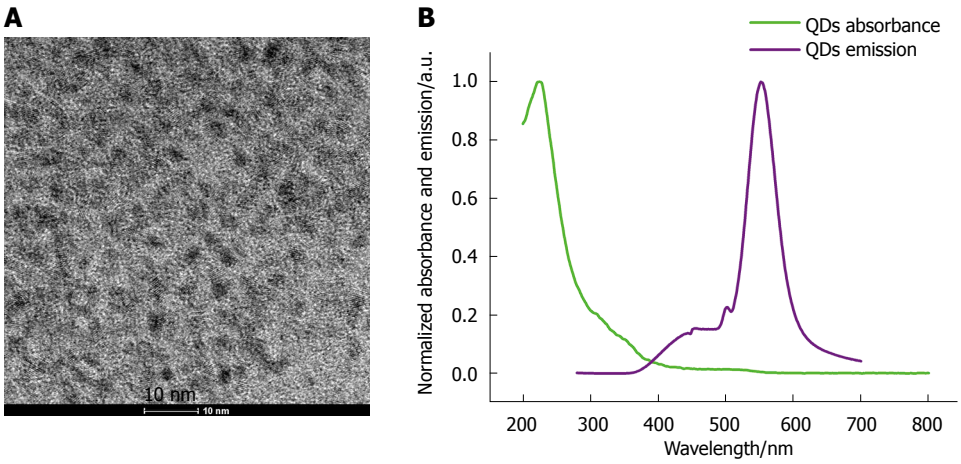


Figure 1 Characterization of CdSe/ZnS quantum dots. A: TEM image of CdSe/ZnS QDs; B: Absorbance and emission of CdSe/ZnS QDs. QDs: Quantum dots.

ROS production in the cytotoxicity of QD-PDT, SW1990 cells were preincubated with 5 mmol/L N-acetylcysteine (NAC) (Sigma-Aldrich), a ROS scavenger, for 1 h before treatment. Cell viability was evaluated by the CCK-8 assay.

Transcriptional analysis of time course in response to QD-PDT

Real-time RT-PCR was performed using 7500/7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, United States) to analyze the apoptosis-related mRNA expression of QD-PDT treated SW1990 cells, such as Bax, Bcl-2 and caspase-3. The primers used in the real-time RT-PCR assay were Bax forward (5′-GGAAGAAGATGGGCTGAGG-3′); Bax reverse (5′-TGTCCCGAAGGAGGTTTATT-3′); Bcl-2 forward (5′-CCGGATCACCATCTGAAGAG-3′); Bcl-2 reverse (5′-AGGGCAAAGAAATGCAAGTG-3′); Caspase-3 forward (5′-AGATGGTTTGAGCCTGAGCA-3′); Caspase-3 reverse (5′-CAGTGCGTATGGAGAAATGG-3′); and GAPDH forward (5′-TGCACCACCAACTGCTTAG-3′); GAPDH reverse (5′-GGATGCAGGGATGATGTTC-3′).

Immunoblotting

Proteins were resolved by SDS-PAGE and blotted onto polyvinylidene difluoride membranes. Anti-Bcl-2 (dilution 1:1000; Cell Signaling Technology, Danvers, MA, United States;), anti-Bax (dilution 1:1000; Cell Signaling Technology), anti-caspase 3 (dilution 1:1000; Cell Signaling Technology), anti-cleaved caspase-3 (dilution 1:1000; Cell Signaling Technology) and anti-β-actin (dilution 1:1000; Cell Signaling Technology) antibodies were used to detect their corresponding proteins followed by anti-rabbit or anti-mouse IgG secondary antibodies (dilution 1:1000; Cell Signaling Technology). Image acquisition was performed with the ChemiDoc XRS+ system (Bio-Rad, Hercules, CA, United States). The optical densities of the protein bands were measured by GS710 Densitometer and analyzed with Quantity One image analysis software (Bio-Rad Laboratories).

Statistical analysis

All experiments were performed in triplicate. The results were expressed as mean ± SD and analyzed by the Student's *t* test with SPSS version 13.0 (SPSS Inc., Chicago, IL, United States). Comparisons among multiple groups of data were analyzed by one-way analysis of variance. $P < 0.05$ was considered statistically significant.

RESULTS

Synthesis and characterization of QDs

QDs were synthesized as previously described. The TEM results showed that QDs were spherical particles with an average size of 5 nm. The peaks of QDs in the UV-Vis analysis showed that the absorbance of QDs was the highest in the UV part of the spectrum, and decreased exponentially when approaching higher wavelengths. The photoluminescence spectra demonstrated that QDs have highest luminescence in the visible part of the spectrum, especially at 560 nm (Figure 1).

Cytotoxicity of QDs

The CCK-8 assay was used to examine the viability of SW1990 cells after different treatments. Cell viability was decreased when the concentration of QDs increased (Figure 2A). Longer incubation time led to lower viability (Figure 2B). Cell viability showed a greater reduction with illumination (Figure 2C). QDs with illumination induced more cytotoxicity in SW1990 cells than QDs alone. More cell damage occurred when the light dose was higher. Illumination alone (10, 20 and 30 J/cm^2) without QDs had limited effects on SW1990 cells (Figure 2C).

The QD-PDT-induced subcellular damage of SW1990 cells was detected by TEM (Figure 3). Under normal conditions, SW1990 cells had a round shape

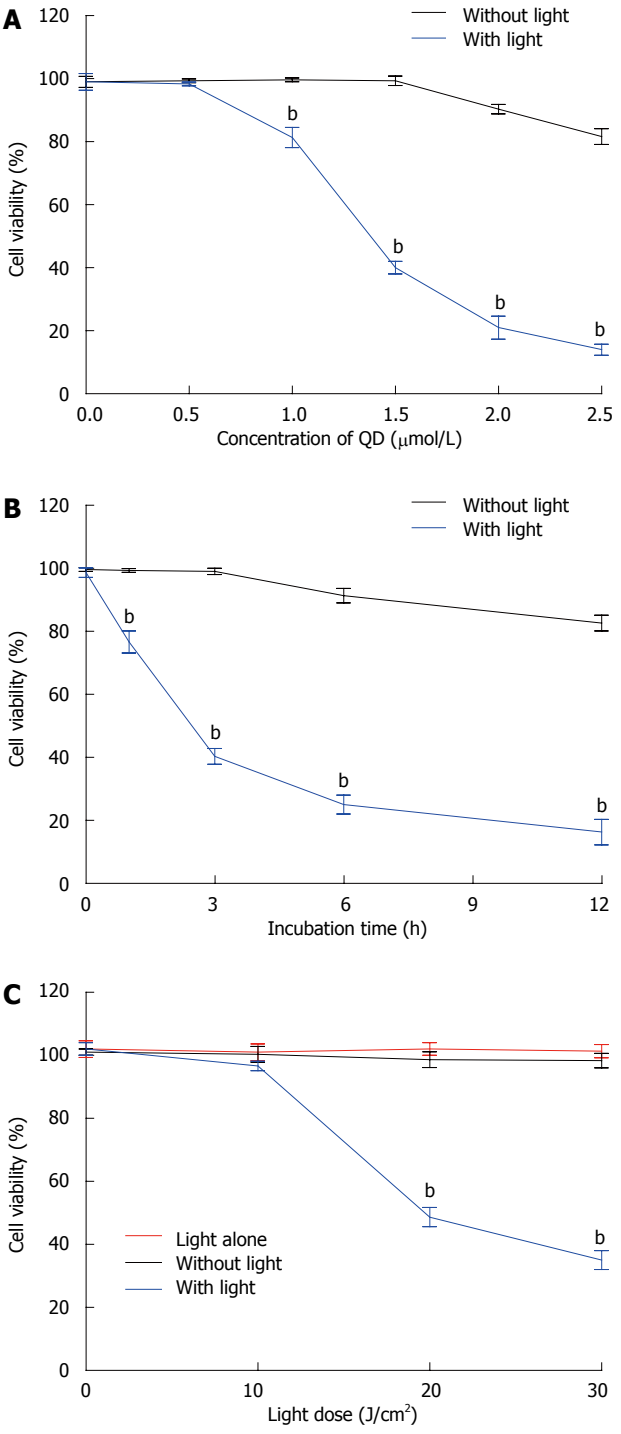


Figure 2 Cell viability of SW1990 cells was inhibited by CdSe/ZnS quantum dots with or without illumination. A: SW1990 cells were treated with various concentrations of CdSe/ZnS QDs (0, 0.5, 1.0, 1.5, 2.0, 2.5 μmol/L); incubation time was 3 h; light dose was 20 J/cm^2; B: SW1990 cells incubated with CdSe/ZnS QDs (1.5 μmol/L) for 0, 1, 3, 6 and 12 h and illuminated (light dose was 20 J/cm^2); C: SW1990 cells alone or incubated with CdSe/ZnS QDs (1.5 μmol/L) for 3 h and illuminated with different light doses (0, 10, 20 and 30 J/cm^2). $^bP < 0.01$ *vs* related group without light. QDs: Quantum dots.

and well-structured mitochondria in the cytoplasm. The cell nucleus was round or class round in the middle of cytoplasm. Nevertheless, after treatment with QDs and illumination, SW1990 cells were significantly

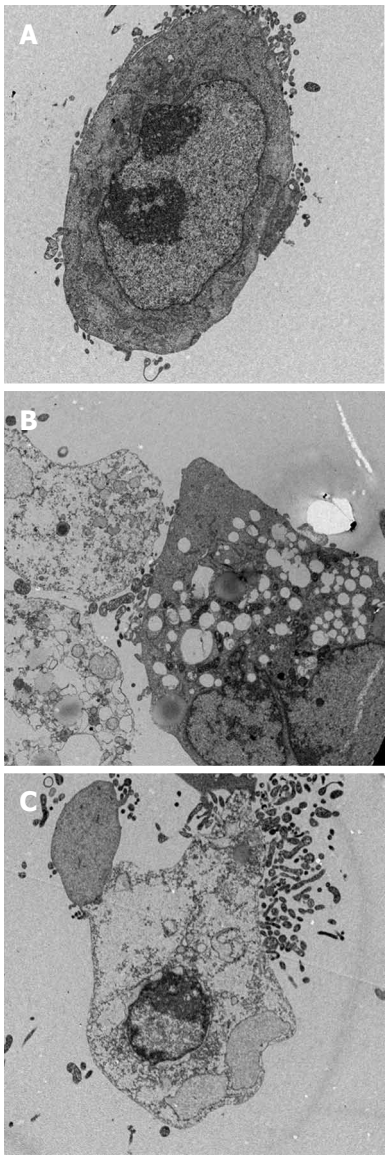


Figure 3 Ultrastructural changes in SW1990 cells induced by CdSe/ZnS quantum dots with or without illumination (TEM, magnification × 2000). A: normal SW1990 cells; B: treated with CdSe/ZnS QDs (1.5 μmol/L, 3 h) and illumination (20 J/cm^2); C: treated with CdSe/ZnS QDs (2 μmol/L, 3 h) and illumination (20 J/cm^2). QDs: Quantum dots.

damaged. Vacuoles and irregularly sized mitochondria appeared. Organelle degeneration, and chromatin condensation and aggregation at the periphery of the nucleus were observed (Figure 3B and C). The main difference between treatment with 1.5 and 2 μmol/L was the percentage of apoptotic and dead cells, thus the latter induced more cell death.

The percentage of apoptotic and necrotic cells was analyzed by fluorescence microscopy and FCM. SW1990 cells were stained with PI and Hoechst 33342. There were more apoptotic bodies in Group D and several cells were even stained with red fluorescence (Figure 4). FCM indicated that the percentage of apoptotic cells was higher in Group D, however, the percentage of necrotic cells remained at a low level

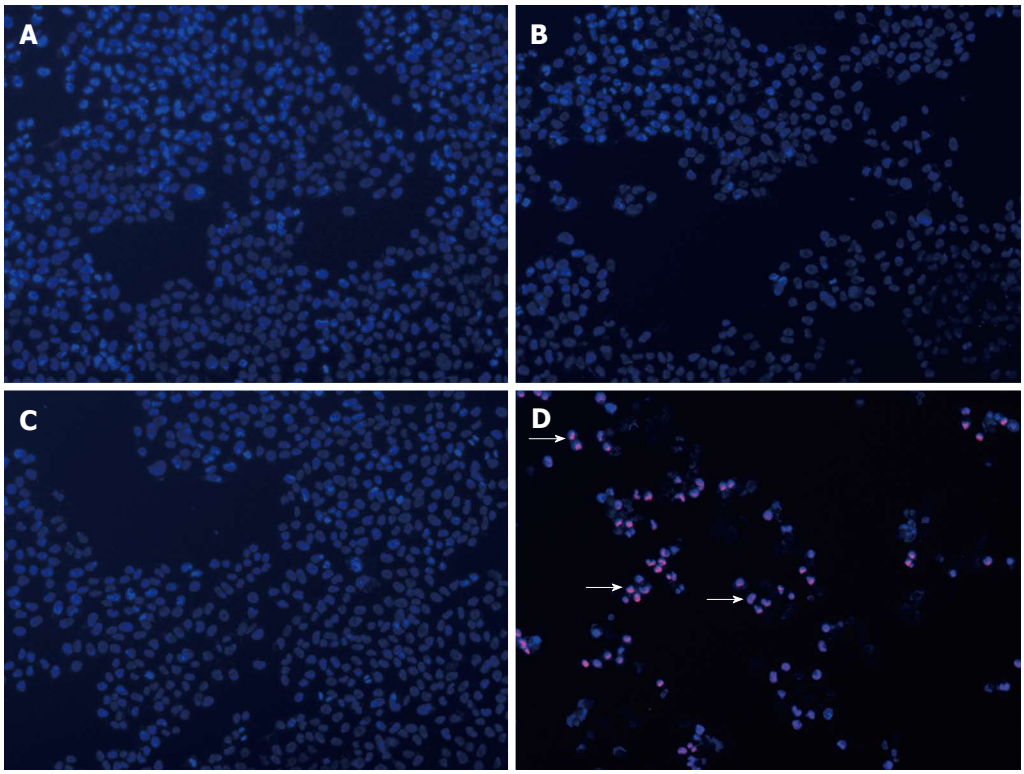


Figure 4 Apoptosis and necrosis were observed in SW1990 cells treated by CdSe/ZnS quantum dots with illumination (Hochest 33342/PI nucleus staining, magnification × 100). A: normal SW1990 cells; B: SW1990 cells with illumination (20 J/cm^2); C: SW1990 cells treated with CdSe/ZnS QDs (1.5 μmol/L, 3 h); D: SW1990 cells treated with CdSe/ZnS QDs (1.5 μmol/L, 3 h) and illumination (20 J/cm^2). The white arrow shows dead cells. QDs: Quantum dots.

(Figure 5).

Measurement of ROS generation

ROS generation was determined by DCF fluorescence in SW1990 cells. The intracellular ROS content in SW1990 cells treated with light (20 J/cm^2) was increased. However, ROS formation significantly increased in SW1990 cells with QDs (1.5 μmol/L, 3 h) and PDT (1.5 μmol/L, 3 h, 20 J/cm^2), especially in cells with PDT (Figure 6A and B). These results indicated that illumination enhanced ROS generation in SW1990 cells treated with QDs.

Expression of mRNA and protein

The mRNA expression level of Bax, Bcl-2 and caspase-3 was measured by RT-PCR. The expression level of each gene was normalized to GAPDH. The mRNA expression level of Bax and caspase-3 increased significantly as compared to control cells, while the level of Bcl-2 decreased (Figures 7 and 8). The protein expression level of these three genes was consistent with corresponding mRNA expression.

Effects of ROS and caspase inhibitors on QD-induced PDT

Pretreatment with an antioxidant (NAC), markedly restored cell viability of SW1990 cells after QD-PDT treatment (Figure 6C), which verified the role of ROS in QD-PDT-induced cytotoxicity. To demonstrate the role of apoptosis in QD-PDT, the pan-caspase inhibitor Z-VAD-FMK was added to the cell culture 1 h before treatment. Inhibition of caspase activation by Z-VAD-FMK abrogated QD-PDT-induced cell death (Figure 9).

DISCUSSION

PDT has been widely used clinically to treat a wide range of malignant cancers, such as esophageal and skin cancer. PDT consisted of two parts: administration of a PS and exposure to light to activate the agent[13,14]. In this study, we synthesized QDs with a CdSe core and ZnS shell and demonstrated the possible QD-induced PDT effects on pancreatic cancer cells.

Selection of an appropriate light wavelength was important. Blue light resulted in inefficient tissue penetration, unlike red and infrared radiation. The range of 600-1200 nm was considered the optical window for tissue penetration. However, only light < 800 nm could generate $^1O_2$, and light > 800 nm could not provide sufficient energy to initiate photosensitization[15]. Thus, there is no ideal single light source for all PDT reactions, even with the same PS. In this study, we selected 365 nm as our illumination wavelength, which happened to be the appropriate excitation wavelength for CdSe/ZnS QDs.

It is reported that cadmium induces ROS generation and triggers apoptosis *via* a caspase-dependent pathway[16,17]. Recently, some studies have shown that

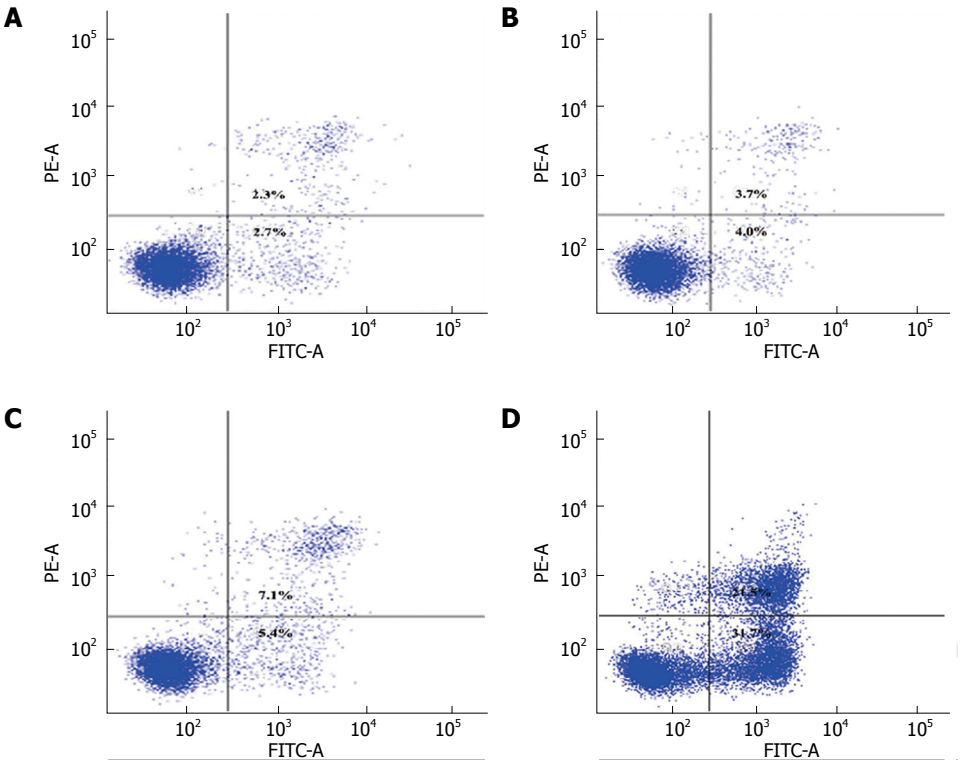
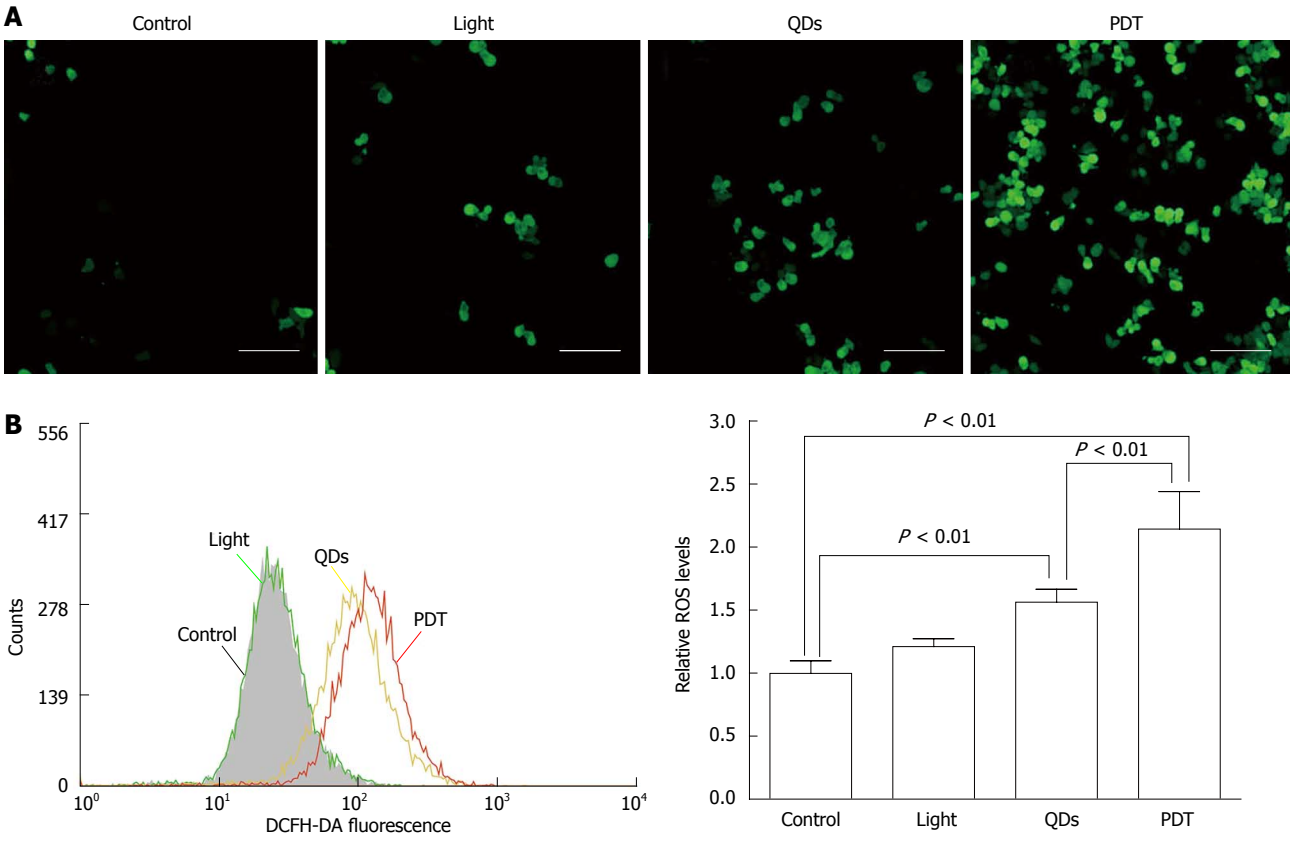


Figure 5 Apoptosis and necrosis were observed in SW1990 cells treated by CdSe/ZnS quantum dots with illumination. A: normal SW1990 cells; B: SW1990 cells with illumination (20 J/cm^2); C: SW1990 cells treated with CdSe/ZnS QDs (1.5 μmol/L, 3 h); D: SW1990 cells treated with CdSe/ZnS QDs (1.5 μmol/L, 3 h) and illumination (20 J/cm^2). FCM of SW1990 cells showed that SW1990 cells had a higher apoptosis rate (53.2%) in Group D than in Groups A, B and C. QDs: Quantum dots.



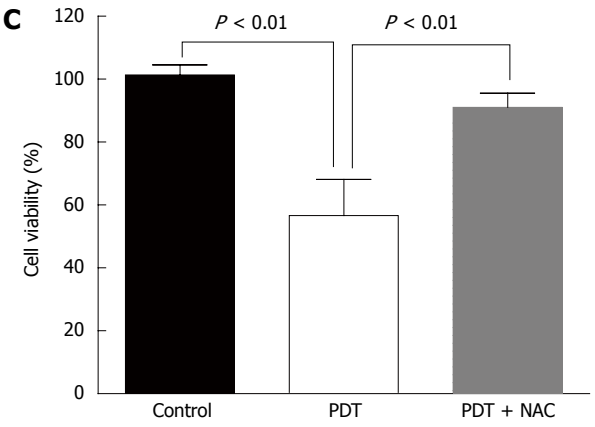


Figure 6 Reactive oxygen species generation was detected after treatment of CdSe/ZnS quantum dots with illumination. A: Fluorescent images of ROS in SW1990 cells (Bar: 200 μm); B: Relative ROS level measured by FCM; C: Cell viability of SW1990 cells by CCK-8 assay. Control: normal SW1990 cells; Light: SW1990 cells with illumination (20 J/cm^2); QDs: SW1990 cells treated with CdSe/ZnS QDs (1.5 μmol/L, 3 h); PDT: SW1990 cells treated with CdSe/ZnS QDs (1.5 μmol/L, 3 h) and illumination (20 J/cm^2). NAC: N-acetylcysteine, a ROS scavenger, 5 mmol/L NAC was added to the cell culture for 1 h before treatment. QDs: Quantum dots; ROS: Reactive oxygen species.

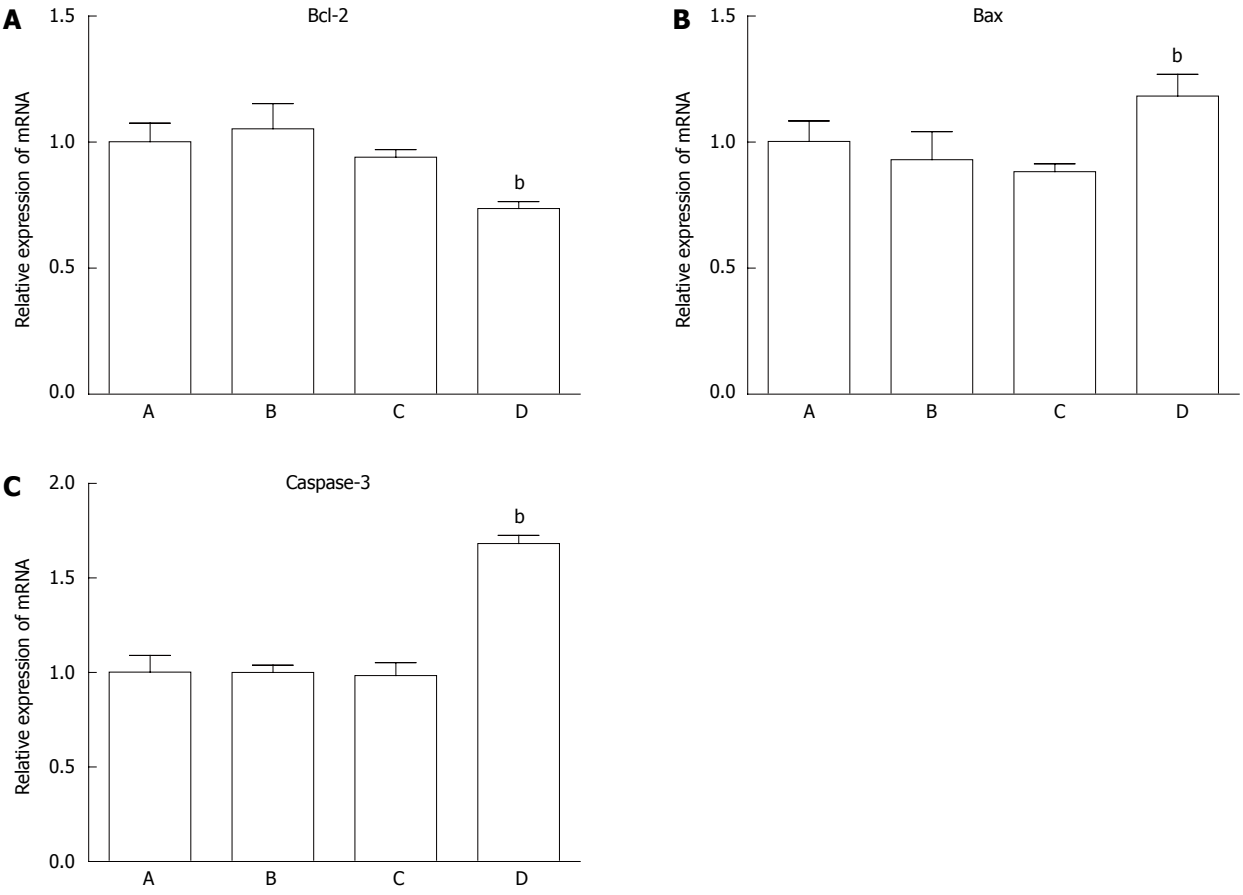


Figure 7 Changes in mRNA expression levels of Bax, Bcl-2 and caspase-3 following CdSe/ZnS quantum dots with illumination. A: Normal SW1990 cells; B: SW1990 cells with illumination (20 J/cm^2); C: SW1990 cells treated with CdSe/ZnS QDs (1.5 μmol/L, 3 h); D: SW1990 cells treated with CdSe/ZnS QDs (1.5 μmol/L, 3 h) and illumination (20 J/cm^2). $^bP < 0.01$ *vs* group A. QDs: Quantum dots.

CdSe-core QDs induce cell death by releasing free Cd^{2+} from the CdSe lattice, and this effect could be impeded by the addition of a coating such as ZnS[18]. Here, we synthesized water-soluble CdSe/ZnS QDs. A ZnS coating made the QDs more biocompatible with cells. However, it effectively reduced ROS generation. QDs are generally used as bioimaging probes for tracing and immunostaining cells[19,20]. In this study, QDs were used as photosensitizers in PDT of the pancreatic cancer cell line SW1990. QDs with ZnS coating showed less cytotoxicity in the dark, even when incubated with cells for 12 h at a concentration of 1.5 μmol/L.

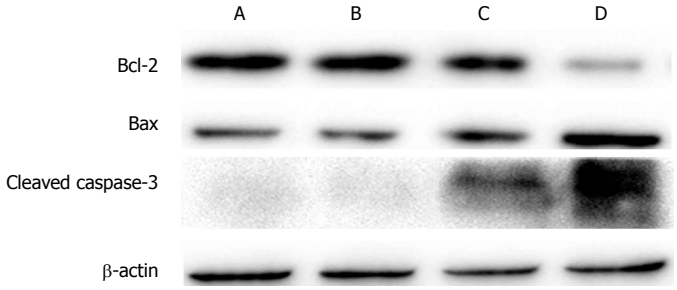


Figure 8 Changes in protein expression levels of Bax, Bcl-2 and caspase-3 following CdSe/ZnS quantum dots with illumination. A: Normal SW1990 cells; B: SW1990 cells with illumination (20 J/cm^2); C: SW1990 cells treated with CdSe/ZnS QDs (1.5 μmol/L, 3 h); D: SW1990 cells treated with CdSe/ZnS QDs (1.5 μmol/L, 3 h) and illumination (20J/cm^2). QDs: Quantum dots.

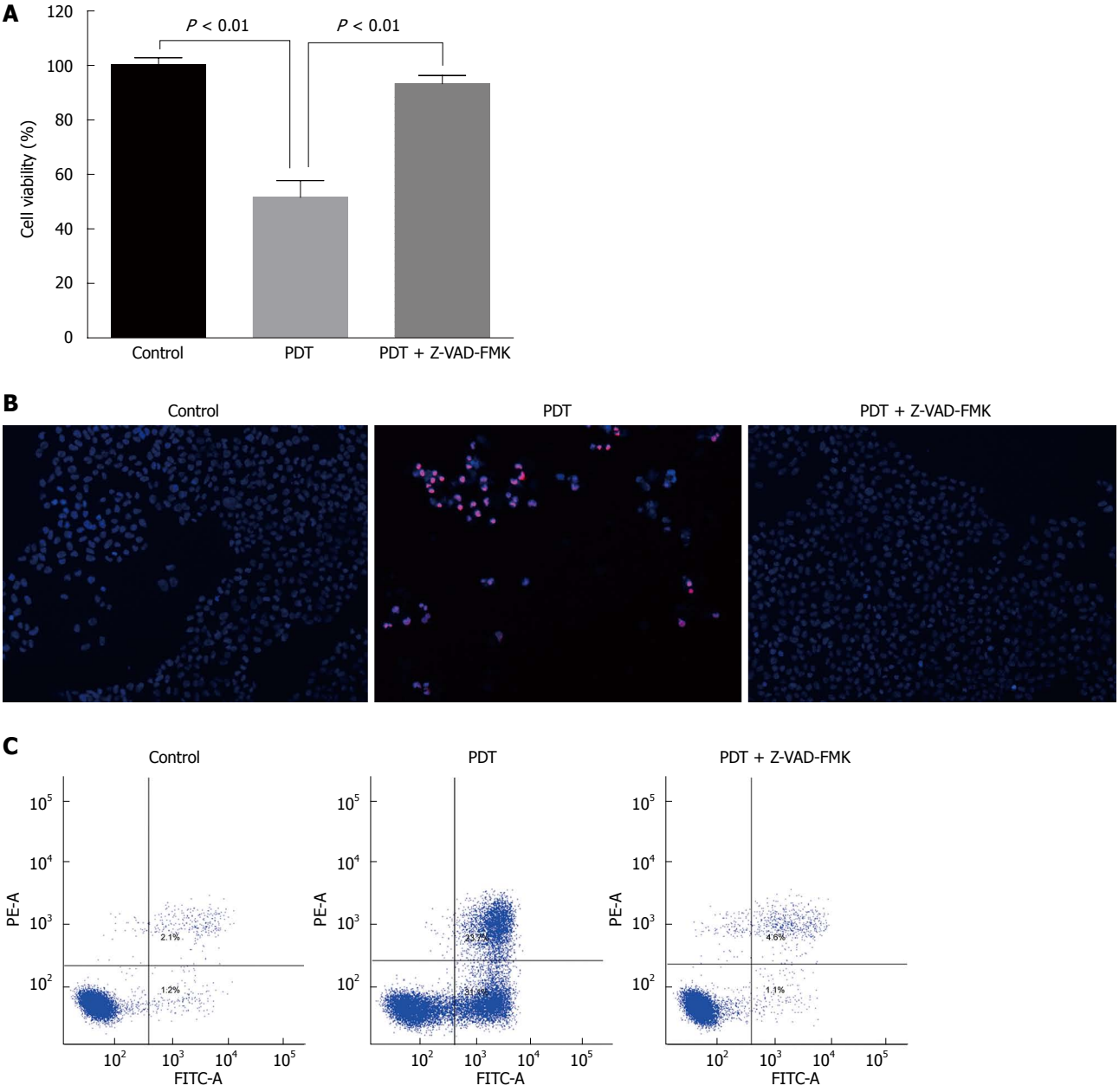


Figure 9 Quantum dots-photodynamic therapy-induced cell death *via* apoptosis. A: Cell viability of SW1990 cells by CCK-8 assay; B: Hochest 33342/PI nucleus staining of apoptotic and necrotic SW1990 cells (magnification × 100), white arrow indicates dead cells; C: Percentage of apoptotic and necrotic SW1990 cells by FCM. SW1990 cells were pretreated with or without Z-VAD-FMK (50 μmol/L) for 1 h and then treated with QD-PDT (1.5 μmol/L, 3 h, 20 J/cm^2). QDs: Quantum dots; PDT: Photodynamic therapy; FCM: Flow cytometry; CCK-8: Cell Counting Kit-8; PI: Propidium iodide.

However, when irradiated by UV, the cytotoxicity of QDs was apparent. Cell viability was decreased when the concentration of QDs increased and incubation time with QDs and light dose increased, which was similar to other studies[21]. TEM, fluorescence microscopy, and FCM illustrated the ability of QDs to generate PDT.

During PDT, ROS generation increased, as reported by Waterhouse *et al*[22], who suggested that the mitochondrion was a vital organelle in programmed cell death and could be mediated by many regulatory factors of apoptosis. To determine whether ROS were increased in QD-induced PDT, we used a probe to detect intracellular ROS variation. Surprisingly, even when coated with ZnS, QDs still generated ROS after illumination, which was statistically significant compared with the control, light and QDs groups. Inhibition of ROS generation with NAC attenuated the cytotoxicity of QD-induced PDT of pancreatic cancer cells, thus ROS were important in this procedure.

To investigate the molecular mechanism of QD-induced PDT, we chose three representative proteins (Bcl-2, Bax and caspase-3) to identify their connection with QD-induced PDT. In this study, we observed apoptosis during QD-induced PDT. Apoptosis has been widely studied and is believed to be triggered by several signals, including a series of proteins[23,24]. The Bcl-2 family of proteins constitutes a central checkpoint[25]. Bax and Bcl-2 are two members of the Bcl-2 family and function as regulatory proteins[26,27]. In this study, we found that QDs increased Bax expression and decreased Bcl-2 expression at the mRNA and protein levels. Several studies have clearly defined Bax as a proapoptotic protein and Bcl-2 as an antiapoptotic protein[28]. In our study, Group D (cells with PDT) showed higher expression of Bax and lower expression of Bcl-2, which to some degree explained the greater apoptosis and necrosis in this group. These results were consistent with other studies[29,30]. Caspase-3 is a member of the cysteine-aspartic acid protease family. As an executioner, caspase-3 is practically inactive until it is cleaved by an initiator caspase when apoptotic signaling events occur. Caspase-3 can be activated in apoptotic cells through extrinsic or intrinsic pathways[31-34]. In this study, cleaved caspase-3 was observed after cells were treated with QDs and illumination. To confirm that apoptosis was involved in the QD-induced PDT effects on pancreatic cancer cells, Z-VAD-FMK was used to restore cell survival and indeed promote cell survival. These results indicated that Bcl-2, Bax and caspase-3 participated in the process of QD-PDT-induced apoptosis. Specifically, QD-PDT downregulated Bcl-2, upregulated Bax, and facilitated caspase-3 cleavage, thus promoting the killing of pancreatic cancer cells.

In summary, this study showed that QDs could be potential PSs for PDT to treat pancreatic cancer by inhibiting SW1990 cell proliferation and inducing apoptosis through ROS generation. QD-PDT may induce apoptosis through ROS-, caspase-3-mediated apoptotic pathways, with upregulation of apoptosis signaling molecules such as Bax and downregulation of Bcl-2. These findings provide a new application for PDT in pancreatic cancer. However, more preclinical and clinical trials should be undertaken before further clinical application.

COMMENTS

Background

Pancreatic cancer is one of the most malignant tumors and has a poor prognosis. Conventional treatments such as surgery, chemotherapy or radiotherapy are still ineffective. Thus, new therapies and drugs are required.

Research frontiers

Photodynamic therapy (PDT) has been used as adjuvant therapy in a wide range of malignant cancers, such as esophageal and skin cancer, with good curative effects. In PDT, only cells in contact with the photosensitizer (PS), light and oxygen are affected, which make it superior to other adjuvant therapies.

Innovations and breakthroughs

Quantum dots (QDs) have large absorption spectra, narrow and symmetric emission bands, and a high molar extinction coefficient, which make them superior to conventional PSs in PDT. In this study, the authors synthesized QDs with a CdSe core and a ZnS shell and demonstrated the inhibitory effect of QD-induced PDT on pancreatic cancer cells. This effect may be due to ROS generation, caspase-3 cleavage and some apoptotic molecular regulation.

Applications

In the present study, QD-induced PDT showed cytotoxicity to pancreatic cancer cells. This reveals a new potential therapeutic strategy for pancreatic cancer.

Terminology

PDT is an innovative treatment that utilizes a photosensitizing agent followed by light exposure to treat certain diseases.

Peer-review

Authors investigated photodynamic effect of CdSe/ZnS QDs on pancreatic SW1990 cancer cells, and concluded that it could be used as a photosensitizer inhibiting SW1990 cells proliferation and apoptotic protein expression regulation. The study is interesting, with convincing results and conclusions.

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World J Gastroenterol 2016 June 7; 22(21): 5023-5032
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

ORIGINAL ARTICLE

Basic Study

Interleukin-22 ameliorates acute severe pancreatitis-associated lung injury in mice

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Author contributions: Qiao YY, Liu XQ and Xu HW designed the experiments; Qiao YY, Liu XQ, Xu CQ, Zhang Z and Xu HW performed the research; Xu CQ and Xu HW contributed new reagents/analytic tools; Qiao YY, Zhang Z and Xu HW analyzed the data; Qiao YY and Liu XQ wrote the paper; Xu CQ and Xu HW revised the manuscript.

Supported by the Shandong Provincial Science and Technology Committee of China, No. 2014GGH218034.

Institutional review board statement: The study was reviewed and approved by Institutional Review Board of the Shandong Provincial Hospital Affiliated to Shandong University.

Institutional animal care and use committee statement: All animal experiments were carried out with approval by an ethics committee in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and further approved by the Institutional Care Committee (Shandong University, Jinan, China).

Conflict-of-interest statement: None of the authors have any possible conflicts of interest.

Data sharing statement: There are no additional data available.

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Received: February 15, 2016
Peer-review started: February 17, 2016
First decision: March 21, 2016
Revised: April 11, 2016
Accepted: May 4, 2016
Article in press: May 4, 2016
Published online: June 7, 2016

Abstract

AIM: To investigate the potential protective effect of exogenous recombinant interleukin-22 (rIL-22) on L-arginine-induced acute severe pancreatitis (SAP)-associated lung injury and the possible signaling pathway involved.

METHODS: Balb/c mice were injected intraperitoneally with L-arginine to induce SAP. Recombinant mouse IL-22 was then administered subcutaneously to mice. Serum amylase levels and myeloperoxidase (MPO) activity in the lung tissue were measured after the L-arginine administration. Histopathology of the pancreas and lung was evaluated by hematoxylin and eosin (HE) staining. Expression of B cell lymphoma/leukemia-2 (Bcl-2), Bcl-xL and IL-22RA1 mRNAs in the lung tissue was detected by real-time PCR. Expression and phosphorylation of STAT3 were analyzed by Western blot.

RESULTS: Serum amylase levels and MPO activity in the lung tissue in the SAP group were significantly higher than those in the normal control group ($P < 0.05$). In addition, the animals in the SAP group showed significant pancreatic and lung injuries. The expression of Bcl-2 and Bcl-xL mRNAs in the SAP group

was decreased markedly, while the IL-22RA1 mRNA expression was increased significantly relative to the normal control group ($P < 0.05$). Pretreatment with PBS did not significantly affect the serum amylase levels, MPO activity or expression of Bcl-2, Bcl-xL or IL-22RA1 mRNA ($P > 0.05$). Moreover, no significant differences in the degrees of pancreatic and lung injuries were observed between the PBS and SAP groups. However, the serum amylase levels and lung tissue MPO activity in the rIL-22 group were significantly lower than those in the SAP group ($P < 0.05$), and the injuries in the pancreas and lung were also improved. Compared with the PBS group, rIL-22 stimulated the expression of Bcl-2, Bcl-xL and IL-22RA1 mRNAs in the lung ($P < 0.05$). In addition, the ratio of p-STAT3 to STAT3 protein in the rIL-22 group was significantly higher than that in the PBS group ($P < 0.05$).

CONCLUSION: Exogenous recombinant IL-22 protects mice against L-arginine-induced SAP-associated lung injury by enhancing the expression of anti-apoptosis genes through the STAT3 signaling pathway.

Key words: Interleukin-22; Acute severe pancreatitis; Lung injury; Anti-apoptosis gene; Signal transducer and activator of transcription 3

Core tip: Interleukin-22 (IL-22) is recognized today as a key player in the antimicrobial defense, regeneration, and protection against damage. However, no reports have described the effects of IL-22 on acute severe pancreatitis (SAP)-associated lung injury. In this study, we found that IL-22 alleviated SAP-associated lung injury in mice by enhancing the expression of anti-apoptosis genes, such as Bcl-2 and Bcl-xL, through the STAT3 signaling pathway. Therefore, IL-22 and the components of STAT3 signaling pathway may be promising targets in the treatment of SAP-associated lung injury.

Qiao YY, Liu XQ, Xu CQ, Zhang Z, Xu HW. Interleukin-22 ameliorates acute severe pancreatitis-associated lung injury in mice. *World J Gastroenterol* 2016; 22(21): 5023-5032 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i21/5023.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i21.5023

INTRODUCTION

Acute lung injury (ALI) is the most common and serious extrapancreatic complication of acute severe pancreatitis (SAP) and also represents a dominant contribution to high morbidity and mortality rates[1]. However, the mechanism underlying the pathogenesis of SAP-induced ALI remains poorly understood. Current therapeutic approaches are limited, and predominantly aimed at symptomatic and supportive treatments. Studies have shown that SAP leads to the overproduction of several cytokines and inflammatory mediators, which initiates and amplifies systemic inflammatory response syndrome (SIRS), resulting in distant organ dysfunction and the development of ALI[2]. The unmet need for therapies against SAP-associated lung injury and paucity of immune response understanding in SAP urge us to explore the role of interleukin-22 (IL-22) and its possible signaling pathway.

IL-22 is a member of the IL-10 cytokine family with epithelial reparative and regenerative properties and is produced by T helper (Th) 22, Th1, and Th17 cells, γδ T cells, natural killer T (NKT) cells, and innate lymphoid cells (ILCs)[3]. Since its discovery in 2000[4], several research laboratories have made great progress in exploring the biology of IL-22 and the role of IL-22 has been identified in numerous tissues, such as the small intestine, liver, colon, lung, kidney, skin, thymus, and pancreas[3,5]. IL-22 exerts its functions by binding to a transmembrane receptor complex that is composed of two different subunits: IL-22 receptor subunit alpha-1 (IL-22RA1) and IL-10R2[6]. IL-22 receptor activation leads to signal transducer and activator of transcription (STAT) 3-mediated proliferative and anti-apoptotic pathway signaling, as well as antimicrobial induction that helps prevent damage and aid tissue repair[5,7]. Treatment with IL-22, *via* the activation of STAT3, alleviates tissue destruction, promotes intestinal epithelial cell proliferation and survival, and accelerates mucosal wound healing during dextran sodium sulfate (DSS)-induced colitis[8], and contributes to the recovery of goblet cell mucus and rapid amelioration of local intestinal inflammation in Th2-mediated colitis[9]. Similarly, IL-22 is a survival factor for hepatocytes in D-galactosamine (GalN)/lipopolysaccharide (LPS)-induced acute liver failure[10] and has a protective role against acute kidney injury induced by ischemia-reperfusion in mice[11]. In addition, IL-22 can also protect mice against acute pancreatitis induced by caerulein and by choline-deficient diet supplemented with DL-ethionine (CDE)[12]. However, no reports have described the effects of IL-22 on SAP-associated lung injury. The purpose of this study was to examine whether IL-22 could protect mice against SAP-associated lung injury induced by L-arginine and its possible signaling pathway.

MATERIALS AND METHODS

Experimental animals

Male Balb/c mice weighing 18-22 g were provided by the Experimental Animal Center of Shandong University (China). All animals were fed laboratory chow, given water *ad libitum*, and maintained in plastic cages at a constant temperature of 23 ℃ ± 2 ℃ and a relative humidity of 55% ± 2% under a 12 h/12 h light-dark cycle for one week prior to performing

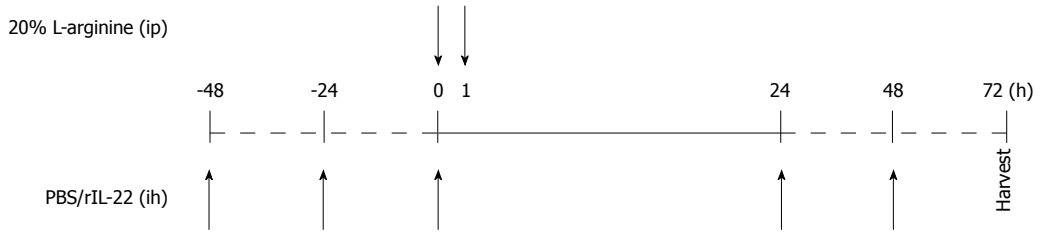

Figure 1 Time points of PBS or recombinant interleukin-22 injection. PBS: Phosphate-buffered saline; rIL-22: Recombinant interleukin-22.

the experiments. All experiments were performed according to the guidelines of the Shandong University Institutional Animal Care and Use Committee (IACUC).

Animal model of SAP and treatments

A total of 72 male Balb/c mice were deprived of food and received only water 12 h before the trial commenced. The mice were randomly assigned to four groups: normal control group (n = 12), SAP group (n = 36), treatment control group (phosphate-buffered saline (PBS) group, n = 12) and treatment group [recombinant IL-22 (rIL-22) group, n = 12]. Mice in the SAP, PBS and rIL-22 groups were injected intraperitoneally (ip) twice with 20% L-arginine hydrochloride (Sigma-Aldrich; pH = 7.0, 4 g/kg bodyweight), at an interval of 1 h. The normal control group received physiological saline injections. PBS or rIL-22 (Miltenyi Biotech) (200 ng/per, 5 times) was administered subcutaneously to mice in the PBS and rIL-22 groups (Figure 1). Mice in the SAP group were killed at 24 h, 48 h, and 72 h after the administration of L-arginine. The remaining mice were sacrificed 72 h after the L-arginine injection.

Serum amylase detection

Mice were thoroughly anesthetized with ether. Orbital blood was collected and stored at -80 ℃ until analysis. Serum amylase levels were measured using an automatic biochemical analyzer.

Determination of myeloperoxidase activity in the lung

The left upper lobe of the lung was harvested and stored at -80 ℃ until assessment. Cryopreserved tissue samples were homogenized, and myeloperoxidase (MPO) activities were measured with MPO detection assay kits following the manufacturer's instructions (Jiancheng Company, Nanjing, China). MPO, a mark of neutrophil accumulation and activation, was expressed as activity units per gram of lung tissue.

Pathological examinations

The head of the pancreas and right upper lobe of the lung were harvested from each mouse and immediately fixed in 10% buffered formalin overnight. Samples were embedded in paraffin wax and cut into 4 μm sections. The sections were flattened, mounted, and heated on blank glass slides. After deparaffinization and dehydration, the sections were stained with hematoxylin and eosin (HE). Pathological examinations were performed by a blinded, unbiased pathologist. The severity of pancreas injury was evaluated based on pancreatic tissue edema, hemorrhage, necrosis and infiltration of inflammation cells. The severity of lung injury was measured according to alveolar congestion, necrosis, hemorrhage, leucocyte infiltration, and thickness of the alveolar membrane.

Real-time polymerase chain reaction

B cell lymphoma/leukemia-2 (Bcl-2), B cell lymphoma/leukemia-extra large (Bcl-xL) and IL-22RA1 mRNA expression levels in the lung tissue were detected by real-time polymerase chain reaction (PCR). Total RNA was isolated from the frozen lung tissue using Trizol reagent (Takara Bio Inc., Japan) following the manufacturer's instructions. RNA purity was tested using a spectrophotometer (Nanodrop Technologies, Wilmington, DE). Reverse transcription-PCR amplification was performed according to the illustrations of the PrimeScript™ RT Reagent Kit (Takara Bio Inc., Japan) with genomic deoxyribonucleic acid (gDNA) Eraser (Perfect Real Time). Real-time PCR was conducted using the Lightcycler480 (Roche). The primer sequences were as follows: Bcl-2: forward, 5′-TGAAGCGGTCCGGTGGATA-3′, reverse, 5′-CAGCATTTGCAGAAGTCCTGTGA-3′; Bcl-xL: forward, 5′-GAGGCAGGCGATGAGTTG-3′, reverse, 5′-ACGATGCGACCCCAGTTT-3′; IL-22RA1: forward, 5′-TCTGGGCTACAAATACATCACCAAG-3′, reverse, 5′-GGCCACTGAGGTCCAAGACA-3′; glyceraldehyde-3-phosphate dehydrogenase (GAPDH): forward, 5′-AAATGGTGAAGGTCGGTGTGAAC-3′, reverse, 5′-CAACAATCTCCACTTTGCCACTG-3′. The cycle conditions were as follows: cDNA denaturation at 95 ℃ for 2 min, followed by 40 cycles of denaturation at 95 ℃ for 10 s, annealing at 60 ℃ for 30 s, and extension at 72 ℃ for 30 s. The specificity of the product was assessed from a melting curve analysis. Results were standardized using GAPDH, and relative amounts were then calculated according to a $2^{-\Delta\Delta CT}$ method.

Western blot analysis

Lung tissue (0.5 g) was ground rapidly in liquid nitrogen to provide 1 mL homogenate (including a protease inhibitor cocktail), which was diluted 20-fold with the radio-immunoprecipitation assay (RIPA)

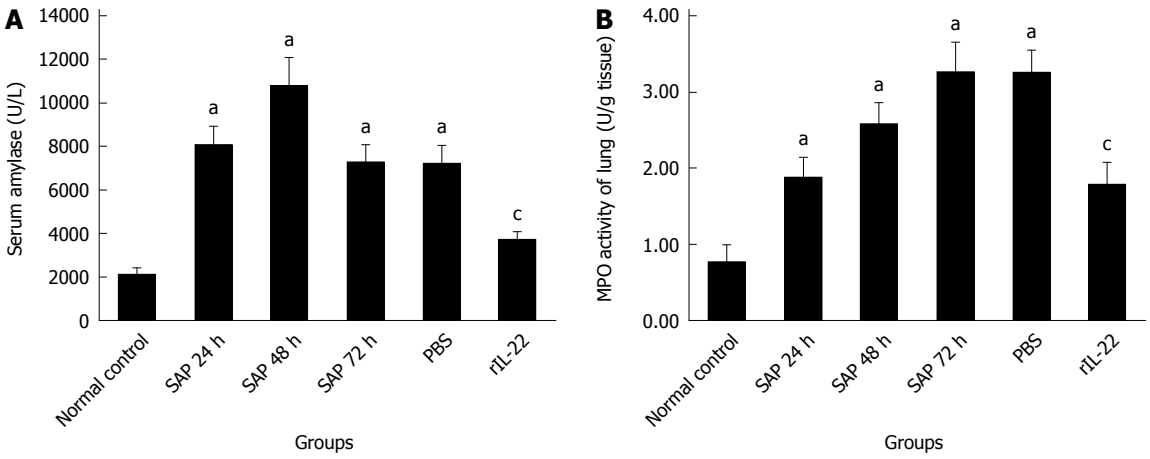

Figure 2 Serum amylase (A) and activity of lung myeloperoxidase (B). Numbers of cases from each group statistically analyzed are 12 (Normal control), 9 (SAP 24 h), 8 (SAP 48 h), 8 (SAP 72 h), 8 (PBS) and 12 (rIL-22). Results are presented as mean ± SD. $^{a}P < 0.05$ *vs* the normal control group, $^{c}P < 0.05$ *vs* the SAP group at 72 h. SAP: Acute severe pancreatitis; MPO: Myeloperoxidase; PBS: Phosphate-buffered saline; rIL-22: Recombinant interleukin-22.

efficient cracking liquid (Beyotime Biotech, China) and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore Biotechnology Inc., United States) after being separated on precast 10% sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE) (Beyotime Biotech, China). The membranes were blocked with 5% non-fat dry milk for 1 h. Primary rabbit monoclonal anti-STAT3 (1:2000) and phospho-STAT3^{Tyr705} (p-STAT3^{Tyr705}) (1:2000) antibodies (Cell Signaling, Beverly, MA, United States) were then added and incubated overnight on a rotating wheel at 4 ℃. The membranes were washed three times with tris-buffered saline with tween-20 (TBST) and incubated with a horseradish peroxidase-conjugated secondary antibody (1:2000) for 1 h at room temperature. Finally, the membranes were detected with an enhanced chemiluminescence reagent (ECL, Millipore Biotechnology Inc., United States). Band densities were measured using ImageJ Analysis Software. β-actin served as an internal control protein.

Statistical analysis

The data are presented as the mean ± SD and were statistically analyzed using SPSS version 20.0 software. The statistical differences between multiple groups were determined using one-way analysis of variance. Comparisons between the two groups were conducted using an unpaired *t*-test. A *P*-value < 0.05 was considered statistically significant. Statistical graphs were generated with GraphPad Prism 5 software.

RESULTS

Serum levels of amylase

As indicated in Figure 2A, compared with the normal control group, the level of serum amylase in the SAP group was increased markedly ($P < 0.05$). The highest level of serum amylase was detected at 48 h after the injection of L-arginine. At 72 h after injection, the serum amylase level partially recovered. No significant decrease was found in the PBS group relative to the SAP group at 72 h after the injection ($P > 0.05$). However, the rIL-22 group was markedly lower than SAP group at 72 h after the injection ($P < 0.05$).

Myeloperoxidase activity analysis

As demonstrated in Figure 2B, a gradual increase over time (24, 48, and 72 h after the injection of L-arginine) was observed in the lung MPO activities relative to the normal control group ($P < 0.05$). Pretreatment with PBS did not significantly affect the MPO activity compared to the SAP group at 72 h after the injection ($P > 0.05$). In contrast, rIL-22 significantly decreased the MPO activity compared with the SAP group at 72 h after the injection.

Histomorphology of the pancreas

Macroscopically, the pancreas was edematous 24 and 48 h after the L-arginine administration. By contrast, the pancreas shrank, with many saponification spots on the omentum majus and mesentery, intestinal cavity expanding and bloody ascites, 72 h after the injection of L-arginine and pretreatment with PBS. Under the light microscope, mice in the SAP group 24 h after L-arginine injection exhibited interstitial edema and infiltration of a small number of neutrophils and mononuclear cells. The acinar architecture and integrity were partially destroyed with focal parenchyma necrosis and hemorrhage. The vascular and pancreatic ductal structures appeared undamaged (Figure 3B). At 48 h, interstitial edema, inflammatory cellular infiltration, parenchyma necrosis and hemorrhage were significantly aggravated (Figure 3C). The severity of pancreatic destruction became maximal 72 h after L-arginine administration. About 70%-80% of the pancreatic acinar cells had been destroyed and replaced by inflammatory and fibrotic cells. The pancreatic ducts were expanded and appeared more

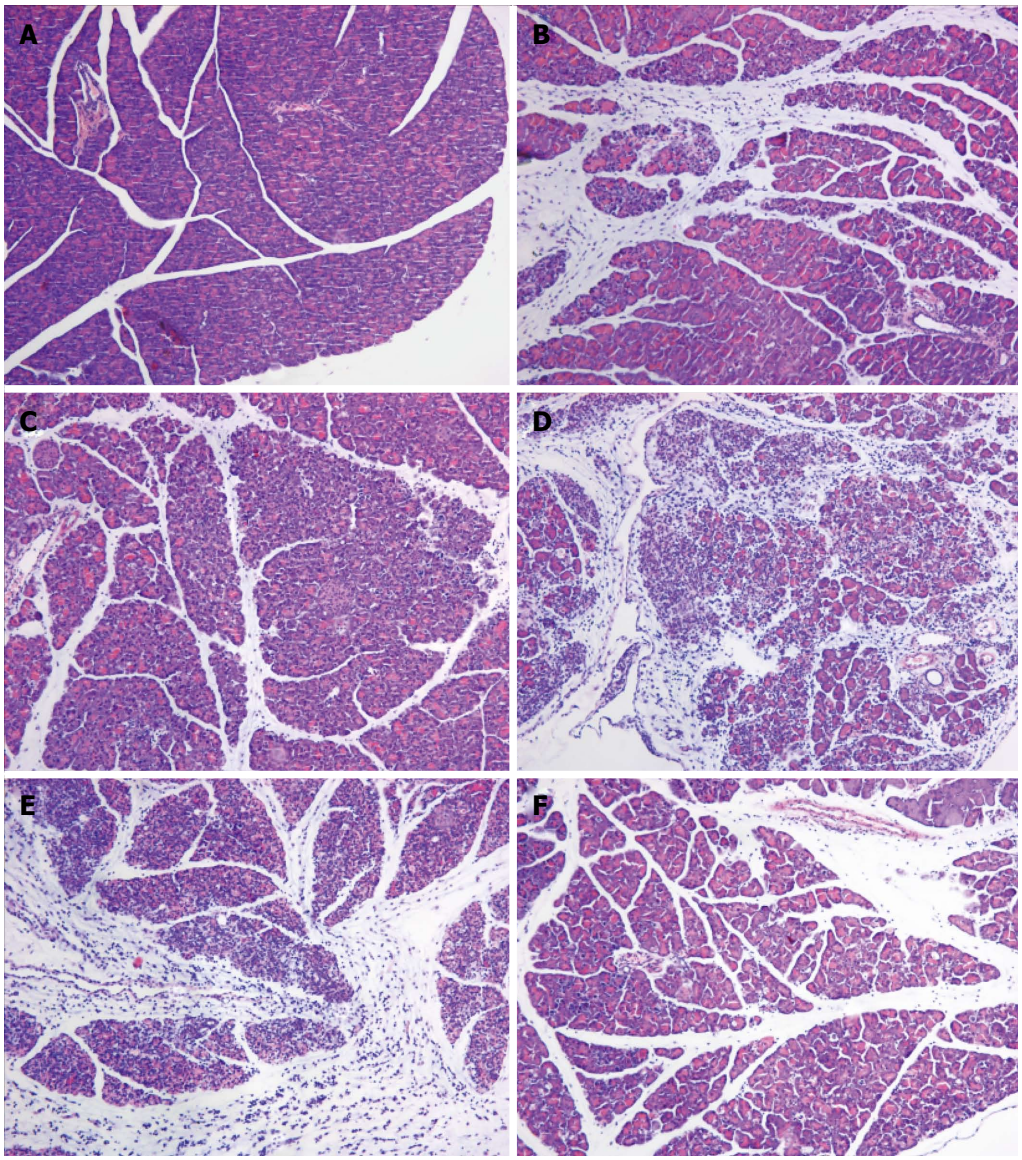

Figure 3 Hematoxylin and eosin staining of the pancreas. Normal control group (A), SAP group (B: 24 h after L-arginine injection; C: 48 after L-arginine injection; D: 72 h after L-arginine injection), PBS group (E) and rIL-22 group (F). Original magnification × 200. SAP: Acute severe pancreatitis; PBS: Phosphate-buffered saline; rIL-22: Recombinant interleukin-22.

numerous because of a decrease in acini and shrinkage of the pancreatic tissue (Figure 3D). Compared with the SAP group, PBS did not significantly reduce the pancreatic injury (Figure 3E). However, the mice in the rIL-22 group showed milder interstitial edema, acinar cell necrosis and cellular infiltration (Figure 3F).

Histomorphology of the lung

The macroscopic view of the lung showed significant edema and hemorrhage in the SAP and PBS groups. By contrast, pulmonary edema and hemorrhage in the rIL-22 group were not obvious. H&E staining showed no observable signs of lung damage in the normal control group (Figure 4A). The lungs of the mice showed no significant swelling, inflammation or necrosis 24 h and 48 h after the L-arginine injection (Figure 4B and D). However, interstitial edema, patchy hemorrhage, thickened alveolar interstitium and infiltration of inflammatory cells were markedly observed 72 h after the L-arginine injection (Figure 4D). No significant difference in the degree of lung injury was found between the PBS and SAP groups at 72 h (Figure 4E). In contrast, pretreatment with rIL-22 significantly reduced the degrees of edema, alveolar congestion and infiltration of inflammatory cells compared with the SAP group at 72 h (Figure 4F).

Bcl-2, Bcl-xL and IL-22R1 mRNA expression in lung tissue

Compared with the normal control group, the expression of Bcl-2 and Bcl-xL mRNAs in the lung tissue in the SAP group showed a significant decrease at 48 h and 72 h after the L-arginine injection ($P < 0.05$). However, no significant decrease was found at 24

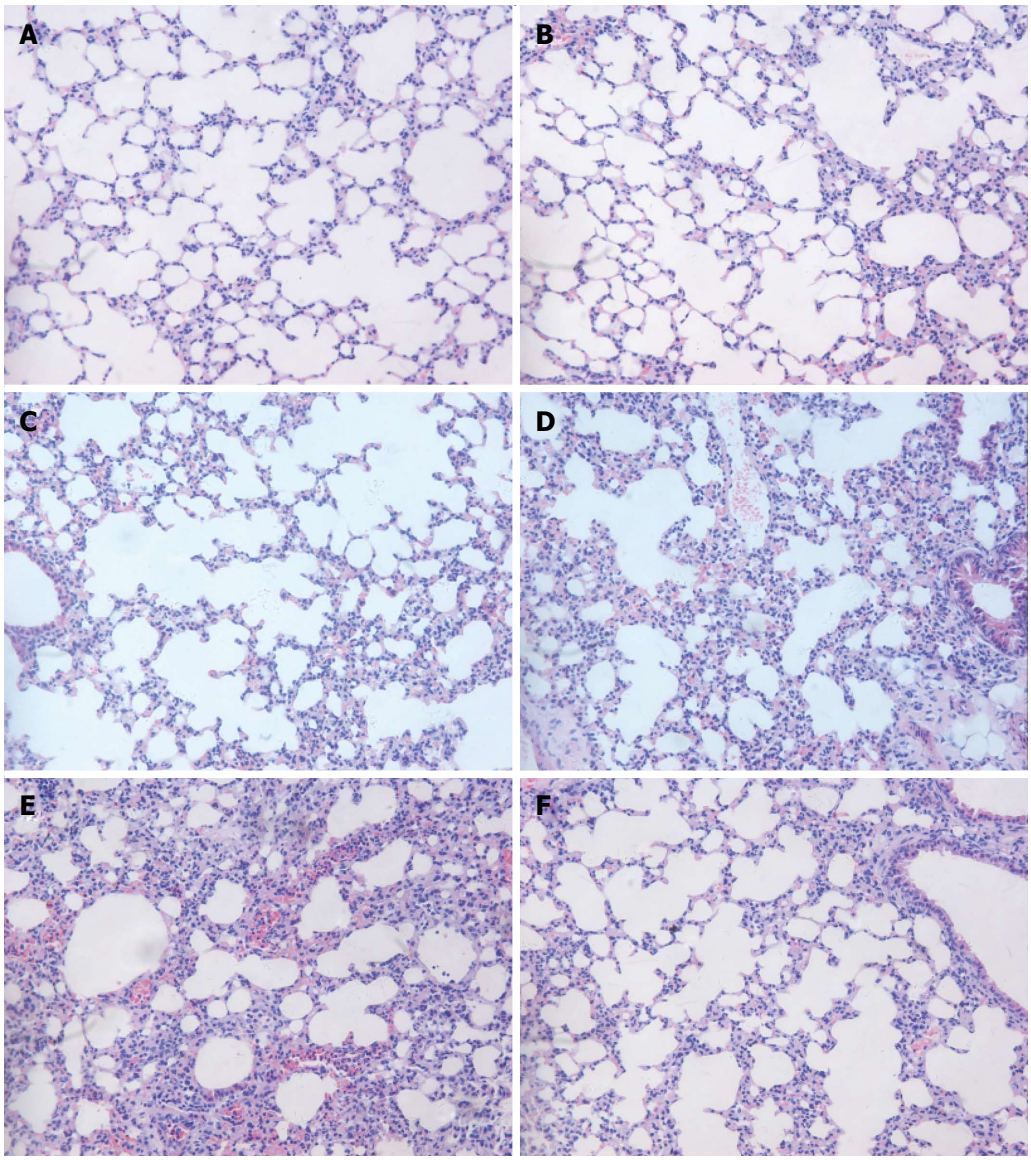

Figure 4 Hematoxylin-eosin staining of the lung. Normal control group (A), SAP group (B: 24 h after L-arginine injection; C: 48 h after L-arginine injection; D: 72 h after L-arginine injection), PBS group (E) and rIL-22 group (F). Original magnification × 200. SAP: Acute severe pancreatitis; PBS: Phosphate-buffered saline; rIL-22: Recombinant interleukin-22.

h after the injection relative to the normal control group ($P > 0.05$, Figure 5A and B). IL-22RA1 mRNA expression significantly increased in the SAP group relative to that in the normal control group ($P < 0.05$). The lowest expression level was detected at 48 h after the L-arginine injection (Figure 5C).

Exogenous IL-22 promotes lung anti-apoptosis gene expression

As demonstrated in Figure 5D, rIL-22 stimulated the expression of Bcl-2 and Bcl-xL mRNAs, which play a key role in preventing cells from apoptosis involved in tissue regeneration ($P < 0.05$). In addition, IL-22RA1 expression in the rIL-22 group was also significantly higher than that in the PBS group ($P < 0.05$).

STAT3 is involved in the protective role of IL-22 in SAP-associated lung injury

STAT3 activation in the lung tissue in rIL-22 group was higher than that in the PBS group. However, there was no significant difference between the two groups as to the STAT3 expression (Figure 6A). The ratio of p-STAT3 to STAT3 protein in the rIL-22 group was significantly higher than that of the PBS group ($P < 0.05$, Figure 6B).

DISCUSSION

SAP is a life-threatening disease characterized by obvious inflammatory reactions and its morbidity has been reported to be increasing continually in

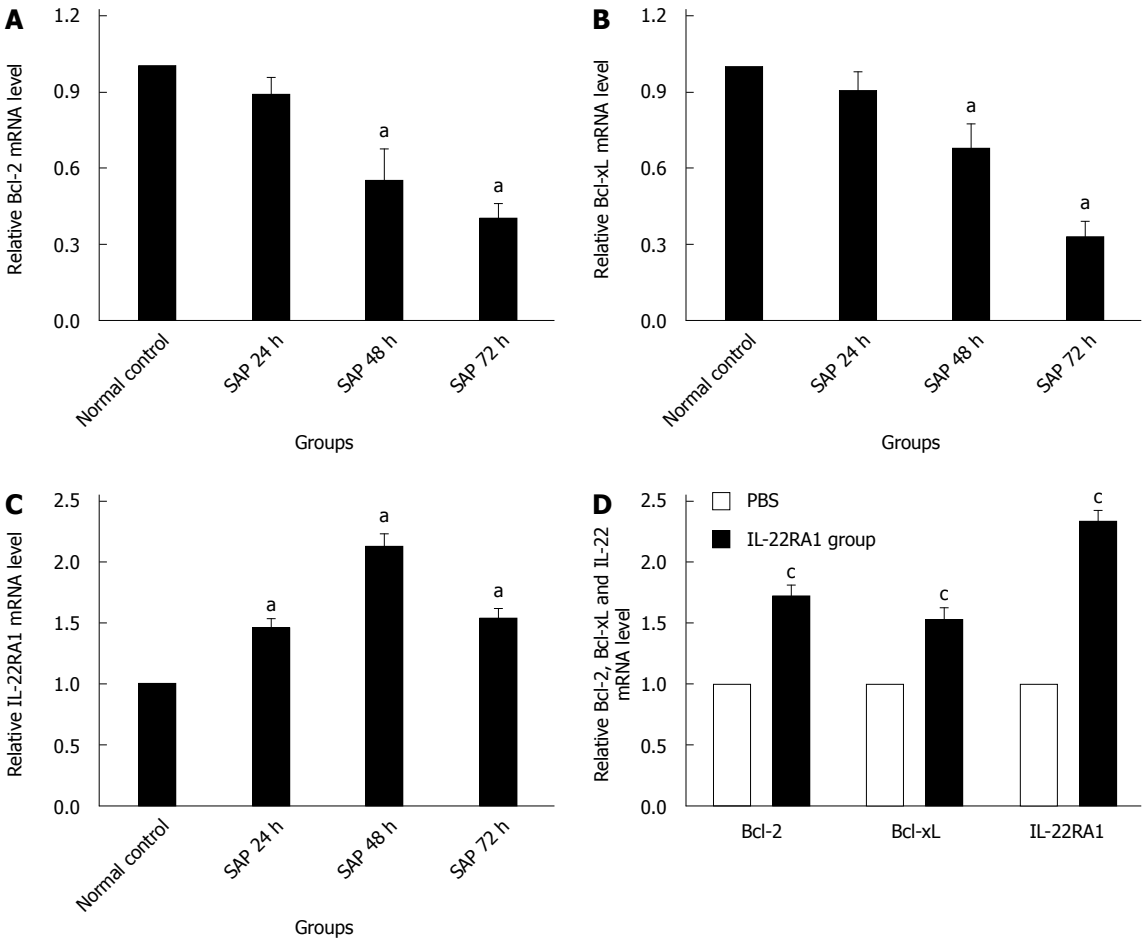

Figure 5 Real-time PCR analysis of expression of Bcl-2(A and D), Bcl-xL (B and D) and IL-22RA1 mRNAs (C and D) in lung tissue. Numbers of cases from each group statistically analyzed are 12 (Normal control), 9 (SAP 24 h), 8 (SAP 48 h), 8 (SAP 72 h), 8 (PBS) and 12 (rIL-22). Results are presented as mean ± SD. $^{a}P < 0.05$ *vs* the normal control group, $^{c}P < 0.05$ *vs* the PBS group. Bcl-2: B cell lymphoma/leukemia-2; Bcl-xL: B cell lymphoma/leukemia-extra large; IL-22RA1: Interleukin-22 receptor subunit alpha-1; PBS: Phosphate-buffered saline; rIL-22: Recombinant interleukin-22; STAT3: Signal transducer and activator of transcription 3.

recent years. Acinar necrosis-induced inflammation can lead to the occurrence of SIRS at an early stage which determines the severity of acute pancreatitis and can induce multiple organ dysfunction syndrome (MODS). ALI is the most prominent extra-pancreatic complication of SAP, and its severity ranges from mild hypoxemia to severe acute respiratory distress syndrome (ARDS) which is responsible for the high mortality rate[1,13]. Recent studies have indicated that the activation of numerous inflammatory cells, such as neutrophils and macrophages, leads to excessive production of cytokines and inflammatory mediators, regulating the severity of acute pancreatitis and SAP-associated ALI[2,14]. An ideal model for studying SAP and its associated multiple organ dysfunction should resemble the course in the human clinical setting, be easily reproducible and severe enough to induce MODS, yet still has a time window long enough for an intervention. In our experiment, the mouse SAP model induced by the L-arginine injection is compatible with the clinical manifestations of SAP, including lung damage. The serum amylase levels and lung tissue MPO activity were increased significantly after the L-arginine administration. In addition, the animals in the SAP group showed obvious pancreatic and lung injuries.

IL-22, formerly named IL-10-related T–cell-derived inducible factor (IL-TIF)[4], has recently gained considerable interest for its tissue protective effects in several murine models. At present, most studies support a well-established protective role for IL-22 in the prevention of hepatocellular damage. Specifically, application of recombinant IL-22 remarkedly attenuates murine liver injury induced by concanavalin A, alcohol, acetaminophen, hepatectomy or ischemia-reperfusion[15-19]. Furthermore, provision of IL-22 also ameliorates high fat diet-induced liver lipogenesis and hepatic steatosis[20] and decreases hepatic fibrosis in mice[21]. In addition to the protective effects of IL-22 on the liver, the benefits of IL-22 application on other targets are also well testified. Administration of IL-22 contributes to the tissue protection and regeneration in murine models of mucocutaneous infection[22-25], ventilator-induced lung injury[26], renal ischemia-reperfusion injury[27] and inflammatory bowel disease[28]. However, it was unknown whether IL-22

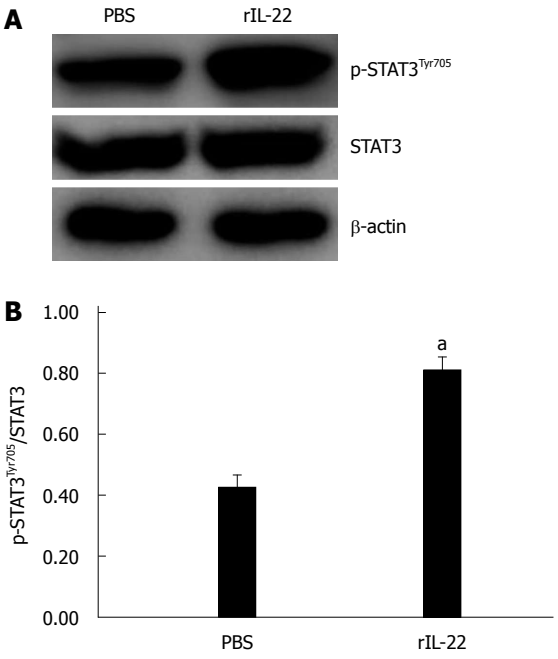

Figure 6 Western blot analysis of expression and activation (Tyr705 phosphorylation) of STAT3 protein in lung tissue (A) and the ratio of p-STAT3^{Tyr705} to STAT3 protein (B). Numbers of cases from each group statistically analyzed were 8 (PBS) and 12 (rIL-22). Results are presented as mean ± SD. $^{a}P < 0.05$ *vs* the PBS group. PBS: Phosphate-buffered saline; rIL-22: Recombinant interleukin-22; STAT3: Signal transducer and activator of transcription 3.

also plays a protective role in SAP-induced ALI. In the present study, we found that the rIL-22-treated mice had significantly lower MPO activity in the lungs than in the untreated SAP mice. Moreover, when compared to the untreated SAP mice, the rIL-22-treated mice showed significantly reduced degrees of edema, alveolar congestion, thickness of the alveolar wall and infiltration of inflammatory cells in the lung injury. Our results are in agreement with the previous findings and demonstrate that rIL-22 may be beneficial to the recovery of SAP-associated lung injury induced by L-arginine.

IL-22 is an important cytokine allowing for cross-talk between leukocytes and epithelial cells because IL-22 production and receptor expression are restricted to leukocytes and epithelial cells, respectively[3,29]. Ligation of the IL-22-IL-22R1-IL-10R2 complex leads to the activation of the JAK1/Tyk2/STAT3 pathway which is known as the principal and dominant mechanism of cellular activation by IL-22[7,8]. Of the cellular tasks associated with STAT3, anti-apoptosis, proliferation and regeneration are the major biological properties of IL-22[30,31]. Apoptosis is recognized as "programmed cell death". Excessive apoptosis probably leads to lung dysfunction in pathological situations. There are increasing data indicating that increased epithelial/endothelial cell apoptosis is involved in the pathogenesis of ALI[32-34]. The signal transduction mechanisms of apoptosis are very complex, and the anti-apoptosis pathway is associated with genes whose functions include "death receptors" and apoptotic regulation. Apoptosis-inducing genes include p53, factor associated suicide (Fas), interlukin-1β-converting enzyme (ICE), reaper (rpr), Bcl-xs, Bcl-2 associated X protein (Bax), Bcl-2 homologous antagonist killer (Bak), Bcl-2/ Bcl-2-XL-associated death promoter (Bad), Bcl-2 inhibitory BH3 domain-containing protein (bid), and Bcl-2 interacting killer (bik). Genes that inhibit apoptosis include Bcl-2, the inhibitor of apoptosis protein (IAP) family, Bcl-xl, Bcl-2 related protein A1 (A1)/Bfl1, Bcl-w, myeloid cell leukemia (Mcl), and Bcl-2-associated athanogene 1 (BAG-1)[35,36]. Bcl-2 is involved in the transduction of the intrinsic mitochondria pathway[37]. IL-22 up-regulates STAT3-inducible proteins, such as Bcl-2, Bcl-xL, cyclin-dependent kinase 4 (CDK4), cyclin D1, cellular-myelocytomatosis viral oncogene (*c-myc*), and p21[15,19,21,38-40], and is associated with tissue protection and regeneration from injury. Along with the activation of extracellular regulated protein kinases (ERK) 1/2[41] and Akt/protein kinase B (PKB)[42], these pro-survival proteins are likely to form the cellular basis for tissue protective characteristics of IL-22. In this study, we examined the expression of IL-22RA1 in the lung of mice with SAP. Interestingly, the level of IL-22R1 expression increased at first and decreased later after the L-arginine administration. This finding indicates that IL-22RA1 expression is stress inducible. Based on the high expression of IL-22RA1, we hypothesized that the lung would respond strongly to an IL-22 administration. To test this hypothesis, we administered rIL-22 systemically to mice and assessed the IL-22 downstream signaling pathway. Consistent with our hypothesis, the lung expression of Bcl-2 and Bcl-xL, downstream targets of STAT3 activation, was decreased significantly in mice with SAP, but was interestingly elevated after rIL-22 treatment. Similarly, IL-22RA1 was also increased after rIL-22 administration. In addition, significant STAT3 activation was observed in the lung after the exogenous IL-22 administration. These data indicate that the mice with SAP-associated lung injury were responsive to rIL-22 administration by enhancing the expression of anti-apoptosis genes through the STAT3 signaling pathway.

Taken together, these findings indicate that systemic administration of rIL-22 can alleviate the SAP-associated ALI in mice by enhancing the expression of anti-apoptosis genes, such as Bcl-2 and Bcl-xL. While the underlying mechanism of IL-22 in protection against SAP-associated lung injury is not fully understood, our findings demonstrate that the STAT3 signaling pathway may be associated with this process. Therefore, IL-22 and the components of STAT3 signaling pathway may be promising targets in the treatment of SAP-associated lung injury.

COMMENTS

Background

Interleukin-22 (IL-22) is recognized today as a key player in the antimicrobial

defense, regeneration, and protection against damage. However, no reports have described the effects of IL-22 on acute severe pancreatitis associated lung injury. In this article, the authors sought to investigate the potential protective effect of exogenous rIL-22 on SAP associated lung injury induced by L-arginine and its possible signaling pathway.

Research frontiers

IL-22 is recognized today as a key player in the antimicrobial defense, regeneration, and protection against damage. However, no reports have described the effects of IL-22 on acute severe pancreatitis associated lung injury.

Innovations and breakthroughs

In this article, the authors investigated the potential protective effect of exogenous rIL-22 on SAP associated lung injury induced by L-arginine and its possible signaling pathway. IL-22 was demonstrated to alleviate acute severe pancreatitis-associated acute lung injury in mice by enhancing the expression of anti-apoptosis genes such as Bcl-2 and Bcl-xL through the STAT3 signaling pathway.

Applications

IL-22 and the components of STAT3 signaling pathway may be promising targets in the treatment of acute severe pancreatitis associated lung injury.

Terminology

IL-22, a member of the IL-10 family, is a cytokine secreted by several types of immune cells such as T helper (Th) 22, Th1, and Th17 cells, γδ T cells, natural killer T cells, and innate lymphoid cells. It is a principal component in mucosal barrier defense, tissue repair, epithelial cell survival, and proliferation.

Peer-review

The authors show that recombinant IL-22 protected the mice against L-arginine-induced SAP and associated lung injury by enhancing the expression of anti-apoptosis genes. The work is very innovative and is an indication for further research on the problem of complications of acute pancreatitis.

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P- Reviewer: Kleeff J, Sliwinska-Mosson M **S- Editor**: Gong ZM
L- Editor: Wang TQ **E- Editor**: Ma S

World Journal of Gastroenterology

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DOI: 10.3748/wjg.v22.i21.5033

World J Gastroenterol 2016 June 7; 22(21): 5033-5041
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

ORIGINAL ARTICLE

Basic Study

^{125}I-labeled anti-bFGF monoclonal antibody inhibits growth of hepatocellular carcinoma

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Supported by the National Natural Science Foundation of China, No. 81273814; and Guangdong Province Key Scientific Research Grant, No. 2013A022100031.

Institutional review board statement: The study was reviewed and approved by the Institutional Review Board of the First Affiliated Hospital of Jinan University, Guangzhou, China.

Institutional animal care and use committee statement: All procedures involving animals were reviewed and approved by the Institutional Animal Care and Use Committee of the Laboratory Animal Center of Jinan University (IACUC protocol number: 20150305001).

Conflict-of-interest statement: The authors declare no potential conflicts of interest related to this paper.

Data sharing statement: No additional data are available.

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Received: February 2, 2016
Peer-review started: February 3, 2016
First decision: March 7, 2016
Revised: March 14, 2016
Accepted: March 30, 2016
Article in press: March 30, 2016
Published online: June 7, 2016

Abstract

AIM: To investigate the inhibitory efficacy of ^{125}I-labeled anti-basic fibroblast growth factor (bFGF) monoclonal antibody (mAb) in hepatocellular carcinoma (HCC).

METHODS: bFGF mAb was prepared by using the 1G9B9 hybridoma cell line with hybridization technology and extracted from ascites fluid through a Protein G Sepharose affinity column. After labeling with ^{125}I through the chloramine-T method, bFGF mAb was further purified by a Sephadex G-25 column. Gamma radiation counter GC-1200 detected radioactivity of ^{125}I-bFGF mAb. The murine H22 HCC xenograft model was established and randomized to interventions with control (phosphate-buffered saline), ^{125}I-bFGF mAb,

^{125}I plus bFGF mAb, bFGF mAb, or ^{125}I. The ratios of tumor inhibition were then calculated. Expression of bFGF, fibroblast growth factor receptor (FGFR), platelet-derived growth factor, and vascular endothelial growth factor (VEGF) mRNA was determined by quantitative reverse transcriptase real-time polymerase chain reaction.

RESULTS: The purified bFGF mAb solution was 8.145 mg/mL with a titer of 1:2560000 and was stored at -20 ℃. After coupling, ^{125}I-bFGF mAb was used at a 1: 1280000 dilution, stored at 4 ℃, and its specific radioactivity was 37 MBq/mg. The corresponding tumor weight in the control, ^{125}I, bFGF mAb, ^{125}I plus bFGF mAb, and ^{125}I-bFGF mAb groups was 1.88 ± 0.25, 1.625 ± 0.21, 1.5 ± 0.18, 1.41 ± 0.16, and 0.98 ± 0.11 g, respectively. The tumor inhibition ratio in the ^{125}I, bFGF mAb, ^{125}I plus bFGF mAb, and ^{125}I-bFGF mAb groups was 13.6%, 20.2%, 25.1%, and 47.9%, respectively. Growth of HCC xenografts was inhibited significantly more in the ^{125}I-bFGF mAb group than in the other groups (P < 0.05). Expression of bFGF and FGFR mRNA in the ^{125}I-bFGF mAb group was significantly decreased in comparison with other groups (P < 0.05). Groups under interventions revealed increased expression of VEGF mRNA (except for ^{125}I group) compared with the control group.

CONCLUSION: ^{125}I-bFGF mAb inhibits growth of HCC xenografts. The coupling effect of ^{125}I-bFGF mAb is more effective than the concomitant use of ^{125}I and bFGF mAb.

Key words: Basic fibroblast growth factor; ^{125}Iodine; Monoclonal antibody; Hepatocellular carcinoma; Fibroblast growth factor receptor; Vascular endothelial growth factor

Core tip: The aim of this study was to investigate the inhibitory efficacy of ^{125}I-basic fibroblast growth factor (bFGF) monoclonal antibody (mAb) in mice with hepatocellular carcinoma (HCC). ^{125}I-bFGF mAb inhibited the growth of HCC xenografts (P < 0.05). The combination of ^{125}I and bFGF mAb was more effective than the concomitant use of ^{125}I and bFGF mAb. ^{125}I-bFGF mAb also significantly reduced the expression of bFGF and fibroblast growth factor receptor (FGFR) mRNA (P < 0.05). Moreover, ^{125}I-bFGF mAb downregulated platelet-derived growth factor mRNA and upregulated vascular endothelial growth factor mRNA.

Hu PH, Pan LH, Wong PTY, Chen WH, Yang YQ, Wang H, Xiang JJ, Xu M. ^{125}I-labeled anti-bFGF monoclonal antibody inhibits growth of hepatocellular carcinoma. *World J Gastroenterol* 2016; 22(21): 5033-5041 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i21/5033.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i21.5033

INTRODUCTION

Hepatocellular carcinoma (HCC) ranks among the most common cancers worldwide. It is the third leading cause of cancer death, with about 700000 cases diagnosed annually[1]. It is characterized by rapid progression, metastasis, and recurrence. Surgical resection and liver transplantation are traditional therapeutic approaches for HCC. Liver transplantation offers many benefits for HCC, but shortage of donor organs and high costs constrain its application. New therapeutic methods, such as radiofrequency ablation, transcatheter arterial chemoembolization, local hyperthermia, and targeted therapy, can also be beneficial to patients with HCC[2-4].

HCC is one of the most vascularized solid tumors, and angiogenesis plays a pivotal role in its development, progression, and metastasis. Basic fibroblast growth factor (bFGF) is one of the most prominent angiogenesis-promoting agents, and its expression closely correlates with tumor angiogenesis[5]. Previous studies have revealed that bFGF stimulates proliferation of human HCC cell lines[6], and the serum bFGF levels in patients with HCC are significantly higher than those in healthy volunteers[7]. These increases in serum bFGF levels correlate closely with HCC invasion and recurrence[8,9]. These studies indicate that specific targeting of bFGF may provide a novel therapeutic strategy for HCC.

bFGF monoclonal antibody (mAb) can specifically bind to bFGF and block its growth-stimulating activity. In our previous studies, we found that bFGF mAb combined with S-1 (gimeracil and oteracil potassium) synergistically inhibited Lewis-transplanted lung cancer, which was related to its inhibition of proliferation and angiogenesis[10]. Combination of bFGF mAb and radiotherapy was shown to exert a synergistic inhibitory effect on the growth of B16-transplanted melanoma tumors, since it increases the radiosensitivity of tumor cells by reducing the expression of bFGF, decreasing angiogenesis, and promoting apoptosis[11]. bFGF mAb also inhibits the proliferation of MCF-7/ADM breast cancer cells and reverses multidrug resistance. The phenomenon may be associated with downregulation of P-glycoprotein and increased intracellular concentration of chemotherapeutic drugs[12].

^{125}I radiotherapy enhances DNA damage, and consequently, induces liver cancer cell apoptosis and improves overall survival in HCC[13]. The use of radionuclide labels on mAbs enhances the specificity of their targeting, and increases the accuracy of evaluating therapeutic response[14]. Thus, coupling bFGF mAb with ^{125}I was used in the present study. Our previous study demonstrated that the half-life of ^{125}I-bFGF mAb

was 81.6-90.3 h and that the radioactive counts were highly detected in the liver tissue of mice[15]. Therefore, ^{125}I-bFGF mAb may be an attractive therapeutic modality for HCC. In this study, we aimed to investigate the feasibility and therapeutic efficacy of ^{125}I-bFGF mAb in HCC.

MATERIALS AND METHODS

Production of bFGF mAb

We prepared the 1G9B9 hybridoma cell line, which was developed in our laboratory with hybridization technology and can secrete mAbs against bFGF. After injecting 10^5 hybridoma cells into each BABL/c mice with incomplete Freund's adjuvant (Sigma-Aldrich, St Louis, MO, United States), ascites was formed in mice 7 d later. The ascites fluid was extracted and purified twice in ammonium sulfate and a Protein G Sepharose affinity column (General Electric, Fairfield, CT, United States). bFGF mAb was identified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The concentration and titer of purified bFGF mAb stock solution were assayed by bicinchoninic acid (BCA) standard assay kit (Pierce, Rockford, IL, United States) and indirectly by enzyme linked immunosorbent assay (ELISA), respectively. Finally, bFGF mAb stock solution was cryopreserved at -20 ℃.

Production of ^{125}I-bFGF mAb

bFGF mAb was labeled with ^{125}I (Amersham Biosciences, Chalfont St. Giles, United Kingdom) by using the chloramine-T method. Afterwards, ^{125}I-bFGF mAb was purified by Sephadex G-25 column (Pharmacia, Piscataway, NJ, United States) in phosphate buffered saline (PBS) (0.05 mol/L, pH 7.5) at room temperature. The labeling efficiency and titer of ^{125}I-bFGF mAb were tested by paper chromatography and indirectly by ELISA, respectively. In order to investigate the stability and storage temperature of ^{125}I-bFGF mAb, assays for radiochemical purity of ^{125}I-bFGF mAb were performed using a gamma radiation counter GC-1200 (Zhongjia Photoelectric Instrument Company, Hefei, China) in 1-8 d with variable temperatures. The radioactive counts of quality controlled samples (0.5, 5.0 and 50.0 ng/mL) were tested by gamma radiation counter GC-1200 in six replicates on three different days to evaluate the accuracy of the assay. The intra-day coefficient of variation (CV) and inter-day CV were also calculated.

Establishment of murine H22 HCC xenograft model

We adjusted the concentration of H22 hepatoma cells to 2.5 × 10^6/mL during the logarithmic growth phase. Each C57BL/6 mouse was injected with 0.2 mL of cells in the armpit of the right front limb. After the tumor diameters grew to 7-8 mm, *Kalium jodatum* was consumed by mice for 3 d to inhibit the absorption of ^{125}I by the thyroid gland before treatment. Twenty-five mice were randomized into five groups: control (PBS), ^{125}I, bFGF mAb, ^{125}I plus bFGF mAb, and ^{125}I-bFGF mAb. The injection doses for each group per mouse were 0.2 mL PBS, 7.4 MBq Na^{125}I, 200 μg bFGF mAb, 7.4 MBq Na^{125}I plus 200 μg bFGF mAb, and 37 MBq/mg ^{125}I-bFGF mAb 200 μg, respectively. The drug was given once every 3 d, five times in total (15 d). After sacrificing the mice and dissecting the tumors, the volume and weight of the tumor were measured and the ratio of tumor inhibition was calculated.

Quantification of bFGF, FGFR, VEGF, and PDGF mRNA expression by quantitative reverse transcriptase real-time polymerase chain reaction

Expression of bFGF, vascular endothelial growth factor (VEGF), fibroblast growth factor receptor (FGFR), and platelet-derived growth factor (PDGF) mRNA was measured by quantitative reverse transcriptase real-time polymerase chain reaction (qRT-PCR). β-actin was used as an internal reference gene. Total RNA was extracted by TRIzol reagent (Invitrogen, Carlsbad, CA, United States). The concentration and quality of the extracted RNA were detected on the measured absorbance at 260 nm and a ratio of (A260/A280). cDNA was synthesized using Transcript High Fidelity cDNA Synthesis Kit (Fermentas, Waltham, MA, United States). The primers of DNA sequences were as follows: bFGF (5'-TAT TTC TTT GGC TGC TAC TTG-3' and 5'-TCC AGC ATT TCG GTG TTG-3'); FGFR (5'-CCT CGT TTG GAG ACG BCT TCA-3' and 5'-GAG CAA AGG GTG TGT GGA CTC T-3'); VEGF (5'-GAA TGT GAT TGC TTT CCT GGG TA-3' and 5'-AGT AAA AGT GGC TGT GGT GGT CCT GA-3'); PDGF (5'-GAG ATA GAC TCC GTA GGG GCT GA-3' and 5'-GAG CAA AGG GTG TGT GGA CTC T-3'); β-actin (5'-CAA GAT CAT TGC TCC TCC TGA-3' and 5'-AGT CCG CCT AGA AGC ATT TG-3'). Using Light Cycler 480 SYBR Green I Master Mix (Roche, Basel, Switzerland), qPCR was performed according to the qPCR protocol. Conditions used for the qPCR amplification were shown as follows: 95 ℃ for 5 min, 55 cycles; 94 ℃ for 10 s, 62 ℃ for 15 s, 72 ℃ for 10 s, and 65 ℃ for 1 min. Melting curves were analyzed to detect the specificity of qPCR products. The expressions of bFGF, VEGF, FGFR, and PDGF mRNA were analyzed by Mx Pro QPCR software version 3.0, and the housekeeping gene β-actin was used as a normalized target gene.

Animal care and use statement

All procedures involving animals were reviewed and approved by the Institutional Animal Care and Use Committee of the Laboratory Animal Center of Jinan University. The animal protocol in our experiment was designed to minimize pain and discomfort to the mice. The mice were acclimatized to laboratory conditions (23 ℃, 12 h/12 h light/dark, 50% humidity, *ad libitum* access to food and water) for 2 wk prior to experimentation. Intragastric administration was carried out with conscious mice, using straight gavage

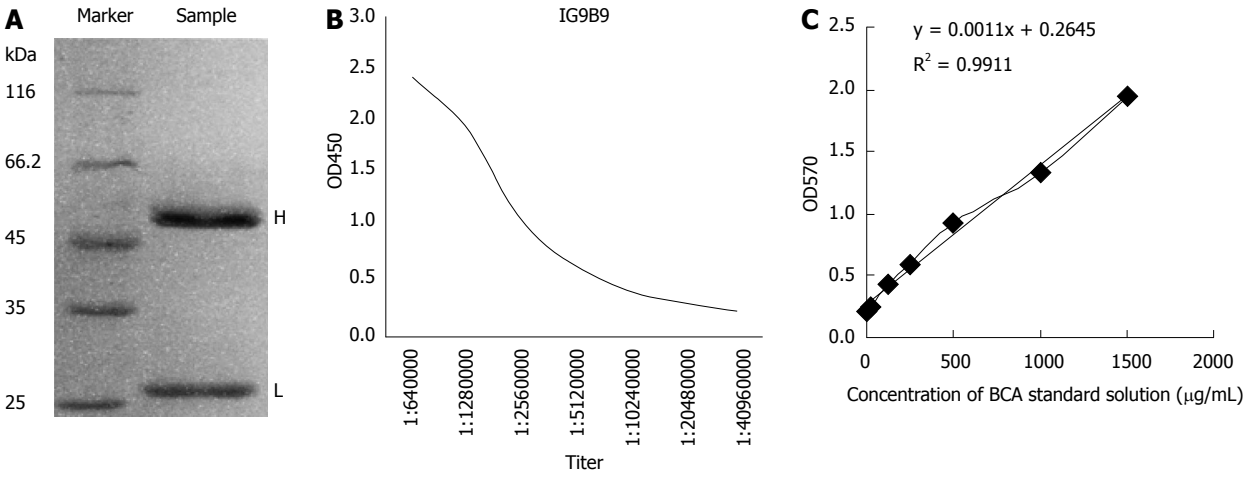


Figure 1 Characterization of anti-bFGF mAb. A: SDS-PAGE of purified bFGF mAb (H: heavy chain; L: light chain); B: Titer of purified bFGF mAb stock solution tested by indirect ELISA; C: Concentration of purified bFGF mAb stock solution assayed by BCA standard assay kit. (Standard curve: y = 0.0011x + 0.2645; Relevancy: R^2 = 0.9911). BCA: Bicinchoninic acid; bFGF: Basic fibroblast growth factor; ELISA: Enzyme-linked immunosorbent assay; mAB: Monoclonal antibody; SDS-PAGE: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

needles appropriate for the animal size (15-17 g body weight: 22 gauge, 2.54 cm length, and 1.25 mm ball diameter). All mice were euthanized by barbiturate overdose (intravenous injection, 150 mg/kg pentobarbital sodium) for HCC xenograft collection.

Statistical analysis

The descriptive data are given as mean and standard deviation. The results were analyzed by SPSS version 16.0 (Chicago, IL, United States) with a *t* test. $P < 0.05$ was considered to be statistically significant.

RESULTS

Product of bFGF mAb

Ascites was produced after 1G9B9 hybrid tumor cells were injected into the abdominal cavity of mice for 7-12 d. Each mouse provided 1.8-2.2 mL ascites fluid, and a final volume of 30 mL was obtained. Based on SDS-PAGE of purified bFGF mAb, there were only two bFGF mAb chains, and there was no non-specific chain, indicating the high purity of bFGF mAb. The molecular weight of the heavy chain was about 50 ku while the light chain was about 25 ku (Figure 1). The titer and concentration of purified bFGF mAb solution were 1:2560000 and 8.145 mg/mL, respectively (Figure 1).

Product of ^{125}I-bFGF mAb

The optimal labeling conditions for the chloramine-T method in our study consisted of chloramine-T 50 μg, Na^{125}I 3.7 MBq, $Na_2S_2O_5$ 100 μg, and bFGF mAb 100 μg, with a reaction time of 45 s. Gamma radiation counter GC-1200 was used to test the radioactivity of the collected tubes. From tubes 1-19, the radioactive counts were close to zero. Starting from tube 20, the radioactivity counts increased and peaked at tube 30. Subsequently, the counts began to decline and reached zero again at tube 46 (Figure 2). Formation of the radioactive peak indicated successful preparation of ^{125}I-bFGF mAb. The remaining liquid was abandoned. The labeling efficiency of ^{125}I-bFGF mAb was ⩾ 90%, which was tested by paper chromatography. The purity of ^{125}I-bFGF mAb became ⩾ 98% after purified by Sephadex G-25 column. The titer was 1:1280000, which implicated no decrease in immunoreactivity (Figure 2). ^{125}I-bFGF mAb was prone to denaturation at room temperature and iodine removal at -20 ℃. ^{125}I-bFGF mAb was stably maintained when stored at 4 ℃ as the level of radiochemical purity remained ⩾ 90% over 6 d. The intra-day CV of quality controlled samples (0.5, 5.0, and 50.0 ng/mL) at the radioactive counts were 0.8%, 1.3%, and 6.8%, respectively, and the inter-day CV was 4.8%, 3.7%, and 8.5%, respectively. The specific radioactivity of the ^{125}I-bFGF mAb used in this study was 37 MBq/mg.

Inhibitory efficacy of ^{125}I-bFGF mAb on HCC

The corresponding volume and weight of the tumor in the control, ^{125}I, bFGF mAb, ^{125}I plus bFGF mAb, and ^{125}I-bFGF mAb groups were 9968 ± 430, 8987 ± 360, 8217 ± 301, 7927 ± 329, and 6210 ± 298 mm^3 and 1.88 ± 0.25, 1.63 ± 0.21, 1.50 ± 0.18, 1.41 ± 0.16, and 0.98 ± 0.11 g, respectively. When compared with the control group, the tumor inhibition ratio in the ^{125}I, bFGF mAb, ^{125}I plus bFGF mAb, and ^{125}I-bFGF mAb groups was 13.6%, 20.2%, 25.1%, and 47.9%, respectively (Figure 3). ^{125}I-bFGF mAb effectively inhibited the growth of HCC ($P < 0.05$), and the tumor inhibition ratio of the ^{125}I-bFGF mAb group was higher than that in the other groups.

Quantitative changes in bFGF, FGFR, VEGF, and PDGF mRNA expression

qRT-PCR amplification and melt curves of β-actin, bFGF, FGFR, VEGF, and PDGF are shown in Figure 4. Expression of these genes entered the plateau of

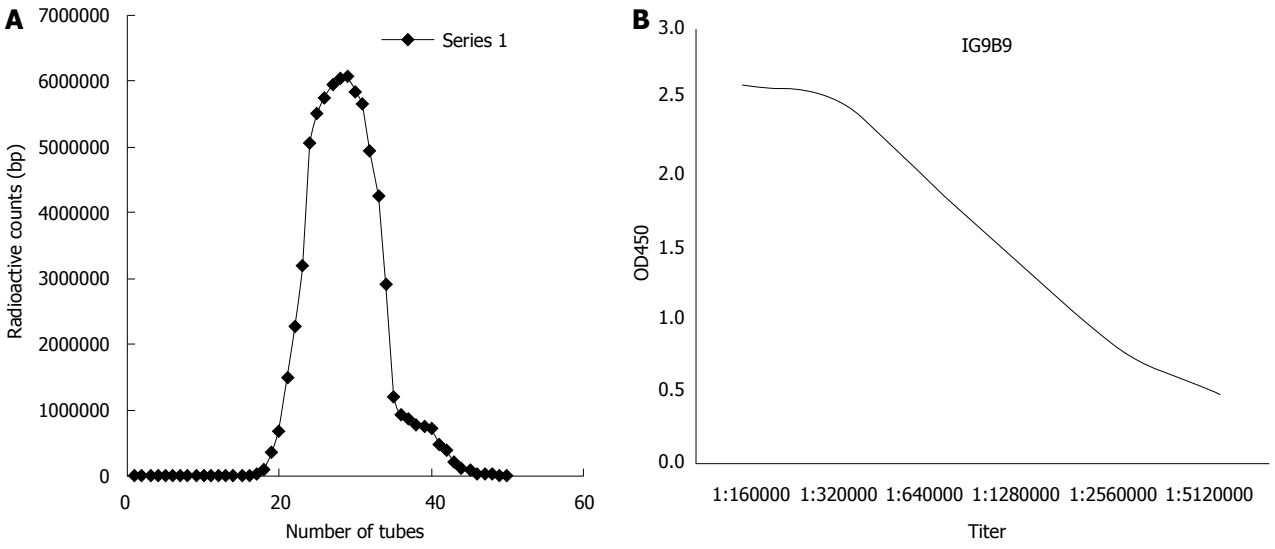


Figure 2 Radioactivity and titer of purified ^{125}I-bFGF mAb. A: Product peak of purified ^{125}I-bFGF mAb tested by gamma radiation counter GC-1200; B: Titer of purified ^{125}I-bFGF mAb stock solution assayed by indirect ELISA.

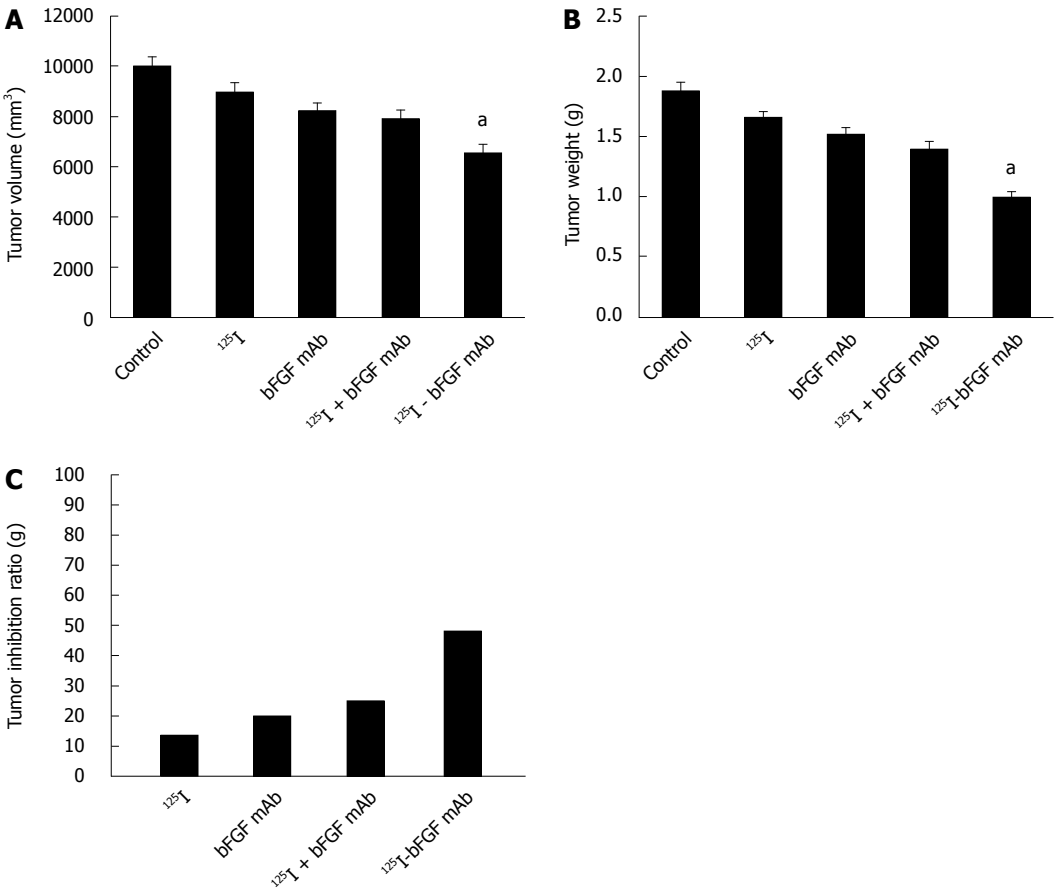
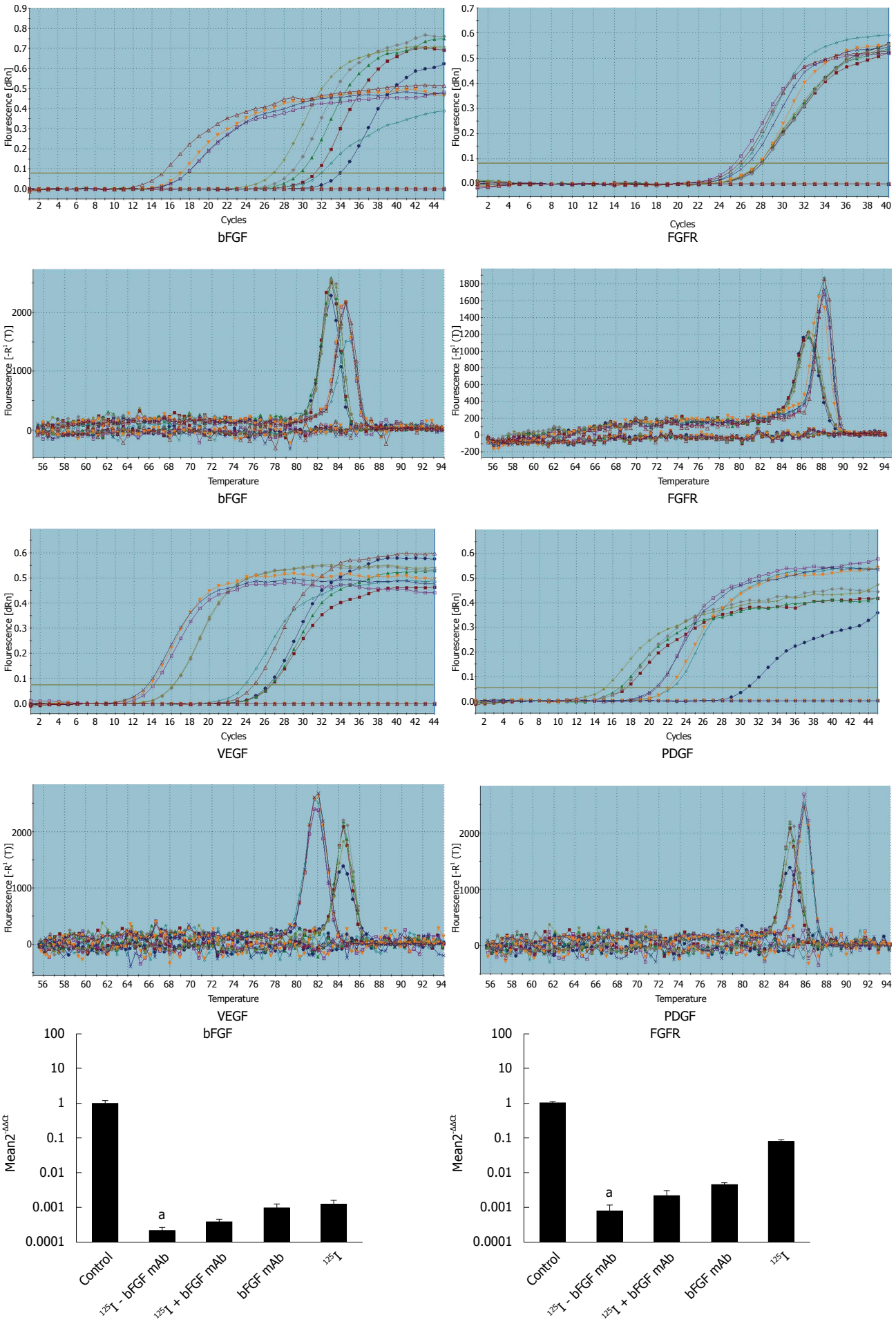


Figure 3 Therapeutic efficacy of ^{125}I-bFGF mAb in mice with H22 hepatocellular carcinoma. A: Tumor volume of H22 HCC; B: Tumor weight of H22 HCC; C: Tumor inhibition ratios of experimental groups; $^{a}P < 0.05$, ^{125}I-bFGF mAb group *vs* other groups (control, ^{125}I, bFGF mAb and ^{125}I plus bFGF mAb). HCC: Hepatocellular carcinoma.

amplification. All the samples were amplified with a single product, and there was no non-specific amplification. According to the relative quantitative method of $2^{-\Delta\Delta Ct}$, the relative expression of bFGF and FGFR mRNA decreased significantly in the ^{125}I-bFGF mAb group when compared with other treatment groups ($P < 0.05$). In groups with interventions, expression of PDGF mRNA decreased while VEGF mRNA was higher (except for ^{125}I group) than that in the control group (Figure 4).


Flourescence [dRn]
Cycles
bFGF
FGFR
Flourescence [-R' (T)]
Temperature
bFGF
FGFR
VEGF
PDGF
Mean2-ΔΔCt
a
Control
125I - bFGF mAb
125I + bFGF mAb
bFGF mAb
125I

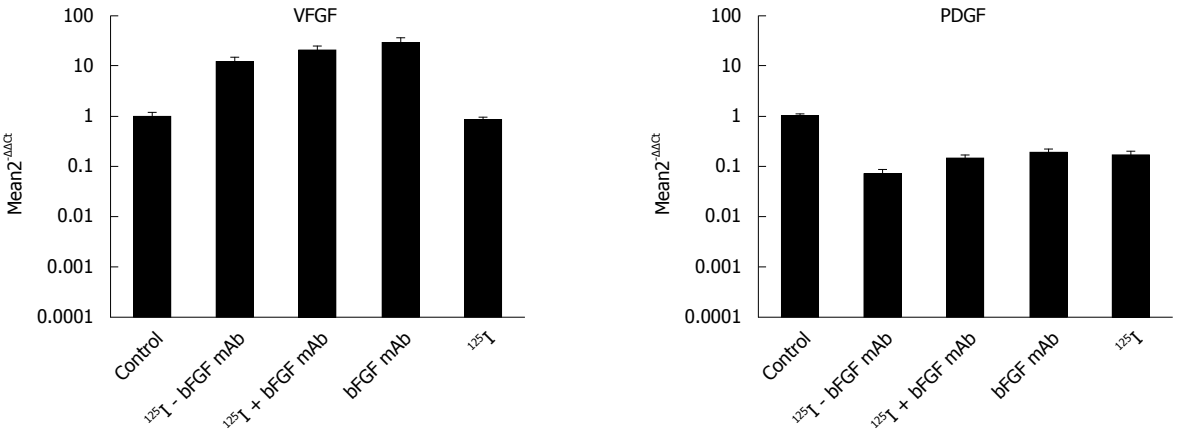


Figure 4 Quantitative real-time reverse transcriptase polymerase chain reaction amplification curves, melt curves and expression of bFGF, FGFR, VEGF and PDGF mRNA. $^{a}P < 0.05$, ^{125}I-bFGF mAb group *vs* other groups (control, ^{125}I, bFGF mAb and ^{125}I plus bFGF mAb). qRT-PCR: Quantitative real-time reverse transcriptase polymerase chain reaction.

DISCUSSION

bFGF is an 18 ku non-glycosylated polypeptide consisting of 146 amino acids that was first isolated and purified from bovine pituitary and brain. It is involved in cell migration and differentiation and is a driving force of mitogenesis and angiogenesis[5]. bFGF has been shown to disrupt the balance of cell cycle progression and apoptosis[16]. By activating protein kinase B, it enhances proliferation of HCC cells *via* the phosphoinositide 3-kinase pathway[17]. Growth of HCC was inhibited by human sulfatase 1, a bFGF-stimulated signaling blocker[16]. Moreover, it was previously demonstrated that a novel mAb to FGF-2 alone, without radiolabeling, effectively inhibited the growth of HCC xenografts[18].

Our results showed that ^{125}I-bFGF mAb significantly inhibited growth of HCC xenografts more than the other interventions ($P < 0.05$) and that the inhibition ratio of the ^{125}I-bFGF mAb group (47.9%) was higher than that of the ^{125}I plus bFGF mAb group (25.1%). Combining ^{125}I and bFGF mAb was more effective than concomitant use of ^{125}I and bFGF mAb in the treatment of HCC. The use of radionuclide labels on mAbs enhanced the specificity of cellular targeting[14]. Such augmented specificity and accuracy could allow ^{125}I-bFGF mAb to yield greater efficacy in treating mice with HCC compared with concomitant use of ^{125}I and bFGF mAb. Among patients with HCC, the serum levels of bFGF were increased, and elevated bFGF independently predicted poor disease-free survival preoperatively[8]. It is tempting to consider ^{125}I-bFGF mAb as a potential clinical option for HCC therapy in the future.

^{125}I-bFGF mAb reduced levels of FGFR and PDGF in our study. FGFR plays a pivotal role in HCC differentiation, proliferation, invasiveness, and resistance to chemotherapy[19-21]. FGFR is highly expressed in HCC and is associated with short overall survival[22]. A humanized monoclonal antibody to FGFR was reported to inhibit tumor growth in HCC xenograft models[23]. In contrast, PDGF, a proangiogenic factor, contributes to vessel maturation[24] and aids in the proliferation and metastasis of HCC[25]. Upregulation of PDGF and PDGF receptors is associated with chemoresistance of gemcitabine and poor prognosis in patients with HCC[26,27]. ^{125}I-bFGF mAb is also a promising agent in tackling liver cancer by decreasing both FGF and PDGF. Perhaps ^{125}I-bFGF mAb enhances therapeutic efficacy of gemcitabine when both agents are indicated in patients with HCC; this possibility requires further investigation.

Our results showed that the expression of VEGF was higher in mice treated with ^{125}I-bFGF mAb, ^{125}I plus bFGF mAb, and bFGF mAb in spite of the improved tumor inhibition ratios and decreased levels of bFGF, FGFR, and PDGF. Previously, increased expression of bFGF was observed in xenotransplanted squamous cell carcinoma after anti-VEGF treatment[28]. Antiangiogenic therapy may impair vessel formation but improve vascular function and tissue oxygenation[29]. Such vessel normalization may become a compensatory reaction of the tumor in response to the depletion of VEGF, leading to increased oxygenation and the observed increased bFGF[28]. In our study, application of anti-bFGF to a murine model of HCC increased VEGF, suggesting that blockade of VEGF elevates bFGF and vice versa. We speculate that vessel normalization also takes place even when anti-bFGF (an antiangiogenic agent) is used and that VEGF is increased by improved tissue oxygenation. Bevacizumab, a potent VEGF inhibitor A, was the first VEGF inhibitor approved by the United States Food and Drug Administration, and it demonstrates modest antitumor activity across a broad range of malignancies when combined with chemotherapy[30]. However, some patients are insensitive to bevacizumab. One study found that a VEGF/bFGF ratio correlated more closely with sensitivity to bevacizumab than with VEGF alone[31]. We found that ^{125}I-bFGF mAb increased

expression of VEGF in the HCC group. Therefore, we hypothesized that ^{125}I-bFGF mAb in combination with VEGF mAb may enhance sensitivity to bevacizumab and improve efficacy in the treatment of HCC. In the future, we will determine the effect of combination ^{125}I-bFGF mAb and bevacizumab on the treatment of HCC.

A recent study found using gefinitib-resistant cell lines that the expression of FGFR1 and bFGF was elevated and that inhibiting either bFGF or FGFR1 by small interfering RNA (siRNA) or FGFR inhibitor (PD173074) restored gefitinib sensitivity. These findings implicate activation of an FGFR autocrine loop as a mechanism of acquired resistance to epithelial growth factor receptor-tyrosine kinase inhibitors (EGFR-TKIs) in non-small cell lung cancer[32]. Since ^{125}I-bFGF mAb can decrease significantly bFGF and FGFR, combining ^{125}I-bFGF mAb and EGFR-TKIs might enhance the therapeutic value of EGFR-TKIs.

In conclusion, ^{125}I-bFGF mAb effectively inhibited the growth of HCC xenografts; significantly reduced expression of bFGF and FGFR; and upregulated VEGF expression. Combined ^{125}I and bFGF mAb was more effective than concomitant use of ^{125}I and bFGF mAb in the treatment of HCC.

COMMENTS

Background

Hepatocellular carcinoma (HCC) is the sixth most common cancer worldwide and the third leading cause of cancer-related death. In clinical practice, the majority of HCC patients are diagnosed at an inoperable stage, resulting in a low long-term survival rate and poor prognosis. Since basic fibroblast growth factor (bFGF) is one of the most prominent angiogenesis-related factors, angiogenesis plays an important role in HCC progression. Here, the authors investigated the biological inhibition efficacy of ^{125}I-labeled bFGF monoclonal antibody (mAb) in mice with HCC. In the near future, these findings might be helpful to clinicians selecting individualized treatment strategies.

Research frontiers

Targeted therapy is one of the main treatment approaches for patients with advanced HCC. New targeted drugs, such as sorafenib and sunitinib, have improved clinical efficacy. However, drug resistance and side effects of sorafenib and sunitinib constrain their clinical application. Therefore, it is necessary to investigate alternative targeting drugs, such as mAb to bFGF, for patients with advanced HCC.

Innovations and breakthroughs

To the best of our knowledge, this is the first study to label bFGF mAb with ^{125}I for the treatment of HCC. The study revealed that ^{125}I-bFGF mAb inhibited growth of HCC xenografts more effectively than the concomitant use of ^{125}I and bFGF mAb. The authors also found that ^{125}I-bFGF mAb reduced expression of bFGF, FGF receptor, and platelet-derived growth factor.

Applications

This study found that ^{125}I-bFGF mAb inhibited growth of HCC xenografts, suggesting that it could be used to tackle liver cancer. More trials are warranted to provide evidence for other applications. ^{125}I-bFGF mAb significantly inhibited the expression of bFGF and FGF receptor, while vascular endothelial growth factor (VEGF) expression was upregulated. Therefore, combination treatment of HCC with VEGF mAb is worthy of further investigation.

Terminology

bFGF mAb is a target drug that can specifically bind to bFGF and block its growth-stimulating activity. It is widely used in laboratory research, and it can significantly inhibit growth of human HCC cell lines *in vitro* and *in vivo*. Therefore, bFGF mAb could be a promising drug in the treatment of liver cancer.

Peer-review

This is a well-designed and executed project on the inhibitory efficacy of ^{125}I-bFGF mAb in HCC. The results show that ^{125}I-bFGF mAb inhibits growth of HCC xenografts more effectively than the concomitant use of ^{125}I and bFGF mAb. ^{125}I-bFGF mAb may be a potential clinical option for HCC therapy in the future.

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P- Reviewer: Giakoustidis A **S- Editor**: Qi Y **L- Editor**: Filipodia
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World J Gastroenterol 2016 June 7; 22(21): 5042-5049
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

ORIGINAL ARTICLE

Basic Study

Transarterial administration of integrin inhibitor loaded nanoparticles combined with transarterial chemoembolization for treating hepatocellular carcinoma in a rat model

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Author contributions: Qian J designed the study and wrote the paper; Qian J, Oppermann E and Tran A performed the majority of experiments and analyzed the data; Qian K performed the nanoparticles preparation; Imlau U performed the molecular investigations; Vogl TJ coordinated the research and reviewed the paper.

Supported by the National Natural Science Foundation of China, No. 81071241.

Institutional review board statement: The study design was approved by Ethics Committee of Johann Wolfgang Goethe University, Frankfurt am Main, Germany.

Institutional animal care and use committee statement: All of the experiments on animals were approved by the German government and the institutional animal research review board, and Jun Qian's license number is Aktenzeichen F86/03.

Conflict-of-interest statement: All authors have seen and agree with the contents of the manuscript and there is no financial interest to report.

Data sharing statement: No additional data are available.

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Received: December 3, 2015
Peer-review started: December 4, 2015
First decision: February 18, 2016
Revised: February 24, 2016
Accepted: March 14, 2016
Article in press: March 14, 2016
Published online: June 7, 2016

Abstract

AIM: To compare the effect of transarterial chemoembolization (TACE) plus GRGDSP (Gly-Arg-Gly-Asp-Ser-Pro, integrin-inhibitor) loaded nanoparticles with TACE alone or TACE + GRGDSP in a rat model of liver tumor.

METHODS: Morris hepatoma 3924A tumors were implanted in the livers of 30 ACI rats. The ACI rats were divided randomly into three groups (10 animals each). Tumor volume before treatment (V1) was examined by magnetic resonance imaging (MRI), and then, after laparotomy and placement of a PE-10 catheter into the hepatic artery, the following interventional protocols were performed: TACE (mitomycin C + lipiodol + degradable starch microspheres) + GRGDSP loaded nanoparticles for group A; TACE + GRGDSP

for group B (control group 1); TACE alone for group C (control group 2). Tumor volume (V2) was assessed by MRI and the mean ratio of the post-treatment to pretreatment tumor volumes (V2/V1) was calculated. Immunohistochemical analysis was performed to assess the quantification of matrix metalloprotein 9 (MMP-9) and vascular endothelial growth factor (VEGF) positive tumor cells in each treatment group.

RESULTS: The mean tumor growth ratios (V2/V1) were 1.3649 ± 0.1194 in group A, 2.0770 ± 0.1595 in group B, and 3.2148 ± 0.1075 in group C. Compared with groups B and C, group A showed a significant reduction in tumor volume. Lower expression of MMP-9 and VEGF in hepatocellular carcinoma was observed in group A than in groups B and C. The angiogenesis of tumor was evaluated using anti-VEGF antibodies, and the metastasis of tumor was assessed using anti-MMP-9 antibody. MMP-9 and VEGF were expressed in all specimens. The immunoexpression of these proteins was confirmed by the presence of red cytoplasmic staining in tumor cells. Lower expression of MMP-9 and VEGF in hepatocellular carcinoma was observed in group A than in groups B and C.

CONCLUSION: Transarterial administration of integrin inhibitor loaded nanoparticles combined with TACE evidently retards tumor growth and intrahepatic metastases compared with TACE alone or TACE plus integrin inhibitor in an animal model of hepatocellular carcinoma.

Key words: Hepatocellular carcinoma; Transarterial chemoembolization; Integrin inhibitor; Nanoparticles; Matrix metalloprotein 9; Vascular endothelial growth factor; ACI rats

Core tip: Our experimental study was designed to reduce tumor progression and recurrence through a combination of transarterial administration of GRGDSP (integrin-inhibitor) loaded nanoparticles plus transarterial chemoembolization (TACE) in an animal model of liver tumor. Our data showed that the combined biological and interventional treatment is a safe and effective therapy compared with TACE alone or TACE plus GRGDSP. The combined multimodal targeting therapies exhibit tremendous advantages over conventional interventional therapy alone.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common malignances worldwide and it has a poor prognosis due to its rapid infiltration, liver cirrhosis and metastases. Surgical resection and liver transplantation are regarded as potentially curative therapies for patients with HCC[1,2]. However, most patients are not suitable candidates for surgical approaches because of liver dysfunction, extrahepatic metastases, lack of donor organs and high recurrence rates. Currently, transarterial chemoembolization (TACE), percutaneous ethanol injection, radiofrequency ablation, microwave coagulation therapy, laser induced thermotherapy and cryotherapy are important components for minimally invasive therapy in patients with cirrhosis and unresectable primary or metastatic liver tumors[3-5]. TACE has been shown to reduce systemic toxicity and increase local effects and thus improve the therapeutic results[6]. However, the long-term survival rate of patients has not been substantiated in randomized clinical studies, mainly due to the tumor recurrence and metastases after treatment[5]. While it is well known that tumor metastasis is a multifactorial process, one key to tumor cell infiltration and metastasis is integrin-mediated adhesion of tumor cells to the normal basement membrane.

Integrin is a king of receptor molecules on the surface of cells, and the basic function of which is to mediate the intercellular adherence or adherence between cells and extracellular matrix (ECM). Integrin expressed by tumor cells and host cells can promote the progress of metastatic dissemination. Recently, studies of anti-integrin therapies are drawing more and more attention to the treatments that protect against recurrence and metastasis of tumors[7]. It was demonstrated that transarterial infusion of GRGDSP (Gly-Arg-Gly-Asp-Ser-Pro integrin-inhibitor which includes RGD-peptide) combined with TACE noticeably inhibited the growth of liver tumor in Wistar rats[8].

It is well known that the nanoparticles considered as drug carriers in the targeting treatment can change the drug distribution in the body, besides the benefit feature of slow drug release. Nanoparticles can be combined with different kinds of drugs or ligands for targeted drug delivery[9,10]. The nanoparticle-therapies have the potential to enhance the effect for inhibiting tumor proliferation and angiogenesis[11,12]. It was reported that the therapeutic effect of chemotherapeutic drug on liver tumor could be noticeably enhanced by the administration of nanoparticles *via* the hepatic artery. The rats that received Adriamycin loaded nanoparticles acquired obvious inhibition on tumor growth, as well as prolonged their survival[11,12]. However, to our knowledge, there have been no experimental or clinical reports on the therapeutic effectiveness of TACE combined with integrin inhibitor-loaded nanoparticles for treatment of HCC. Thus, the purpose of our study

was to assess the effect of TACE combined with GRGDSP loaded nanoparticles, compared with TACE alone or TACE plus GRGDSP for treating HCC in an animal model.

MATERIALS AND METHODS

Tumor cells and animal model

Morris hepatoma 3924A tumors, poorly differentiated HCC, was used in this study. The hepatoma cells were obtained from the German Cancer Research Center in Heidelberg. Thirty male ACI rats (200-220 g) were obtained from Harlan Winkelmann (Borchen, Germany). The experiments were performed in accordance with the German government and the institutional animal research review board. All the experiments were carried out under intraperitoneal anesthesia with ketamine hydrochloride (100 mg/kg), xylazin hydrochloride (15 mg/kg), and atropine sulfate (0.1 mg/kg).

Tumor implantation (day 0) was performed according to the method described by Yang *et al*[13] with slight modification[14]. The tumor tissue was recovered from an animal 12 d after subcutaneous implantation (5×10^6 tumor cells) and cut into small cubes (ca. 2 mm). The left lateral lobe of the liver of the recipient rat was exposed through a subxiphoid abdominal incision and a small subcapsular incision was made. The tumor fragment was gently embedded into the pocket and the abdominal wall was subsequently closed.

Agents

GRGDSP loaded nanoparticles were kindly provided by School of Life Science and Technology, Huazhong University of Science and Technology (Wuhan, China). GRGDSP loaded nanoparticles were synthesized using the method of Yang *et al*[15] with slight modifications. Superparamagnetic iron oxide (SPIO) was used as RGD (Arg-Gly-Asp) nanocarriers. The size and size distribution of the final product were determined by photon correlation spectroscopy (PCS) with a nano-ZS90 laser particle analyzer (Malvern Instruments Corp., United Kingdom). The mean diameter of particles was 107 nm, and the drug loading ratio was 50%.

A dose of 0.25 mg GRGDSP loaded nanoparticles was suspended in 0.6 mL OF 0.9% NaCl for 10 min before administration.

A dose of 0.1 mg mitomycin, 0.1 mL lipiodol and 5.0 mg degradable starch microspheres was administered into the hepatic artery of the rats in the experiment.

MR imaging (days 12 and 25)

One day before and 12 d after the interventional therapy, MRI was performed with a 3.0 Tesla Magnetom superconducting system (Siemens; Erlangen, Germany) using a wrist coil. MR images of the liver were acquired in the transverse plane using a T2-weighted turbo spin-echo sequence with the following imaging parameters: TR/TE, 3870/80 ms; slice thickness, 2 mm; matrix, 192 × 256. The tumor volume was evaluated in T2-weighted images according to the ellipsoid volume formula[16]: $V = 0.5 \times d1 \times d2^2$ (d1 = maximum diameter of the tumor; d2 = minimum diameter perpendicular to d1).

Interventional procedures (day 13)

A second laparotomy was performed 1 d after MRI examination for interventional treatment. A PE-10 polyethylene catheter (inner diameter 0.28 mm, outer diameter 0.61 mm, Wenzel, Heidelberg, Germany) was used for catheterization under a microscope. The catheter was inserted retrogradely into the gastroduodenal artery and pushed to the common hepatic artery. The following therapeutic agents were injected through the catheter to the hepatic artery by sandwich technique (sequential injection of lipiodol + mitomycin + GRGDSP loaded nanoparticles or GRGDSP + degradable starch microspheres):

Group A (TACE + GRGDSP loaded nanoparticles; n = 10): 0.1 mg mitomycin + 0.1 mL lipiodol + 5.0 mg degradable starch microspheres + 0.25 mg GRGDSP loaded nanoparticles.

Group B (control group 1, TACE + GRGDSP; n = 10): 0.1 mg mitomycin + 0.1 mL lipiodol + 5.0 mg degradable starch microspheres + 0.25 mg GRGDSP (2.5 mg/mL, Jingmei Biological, Wuhan, China).

Group C (control group 2, TACE alone; n = 10): 0.1 mg mitomycin + 0.1 mL lipiodol + 5.0 mg degradable starch microspheres.

Immunohistochemical examination (day 26)

All rats were sacrificed after the MRI examination by intravenous administration of overdose sodium pentobarbital. Liver samples were embedded and frozen in Tissue-Tek and 5 μm cryosections were generated. Sections were fixed in 100% acetone and endogenous peroxidase activity was blocked with 0.6% H_2O_2/MeOH followed by incubation with anti-MMP-9 rabbit polyclonal antibody (Cell Signaling Technology Inc., MA, United States) and/or anti-VEGF rabbit polyclonal antibody (Santa Cruz Biotechnology Inc., United States) overnight at 4 ℃. Sections were then incubated with an anti-rabbit alkaline phosphatase supervision polymer system (DCS Innovative Diagnostik-Systeme, Hamburg, Germany), and endogenous alkaline phosphatase was inhibited by 1 mmol/L levamisole present in the substrate. Sections were subsequently counterstained with hematoxylin and mounted in Kaisers Glycerol Gelatin (Merck, Darmstadt, Germany). To evaluate the expression of MMP-9 and VEGF, all slides were examined and scored by two independent pathologists who were blinded to the animal data. Stained cells were counted in 10 microscopic fields (× 100) per slide in tumor area and the average was calculated. Slides were evaluated in a semiquantitative method relating to the percentage staining of the cells and were scored as follows: 0 (No

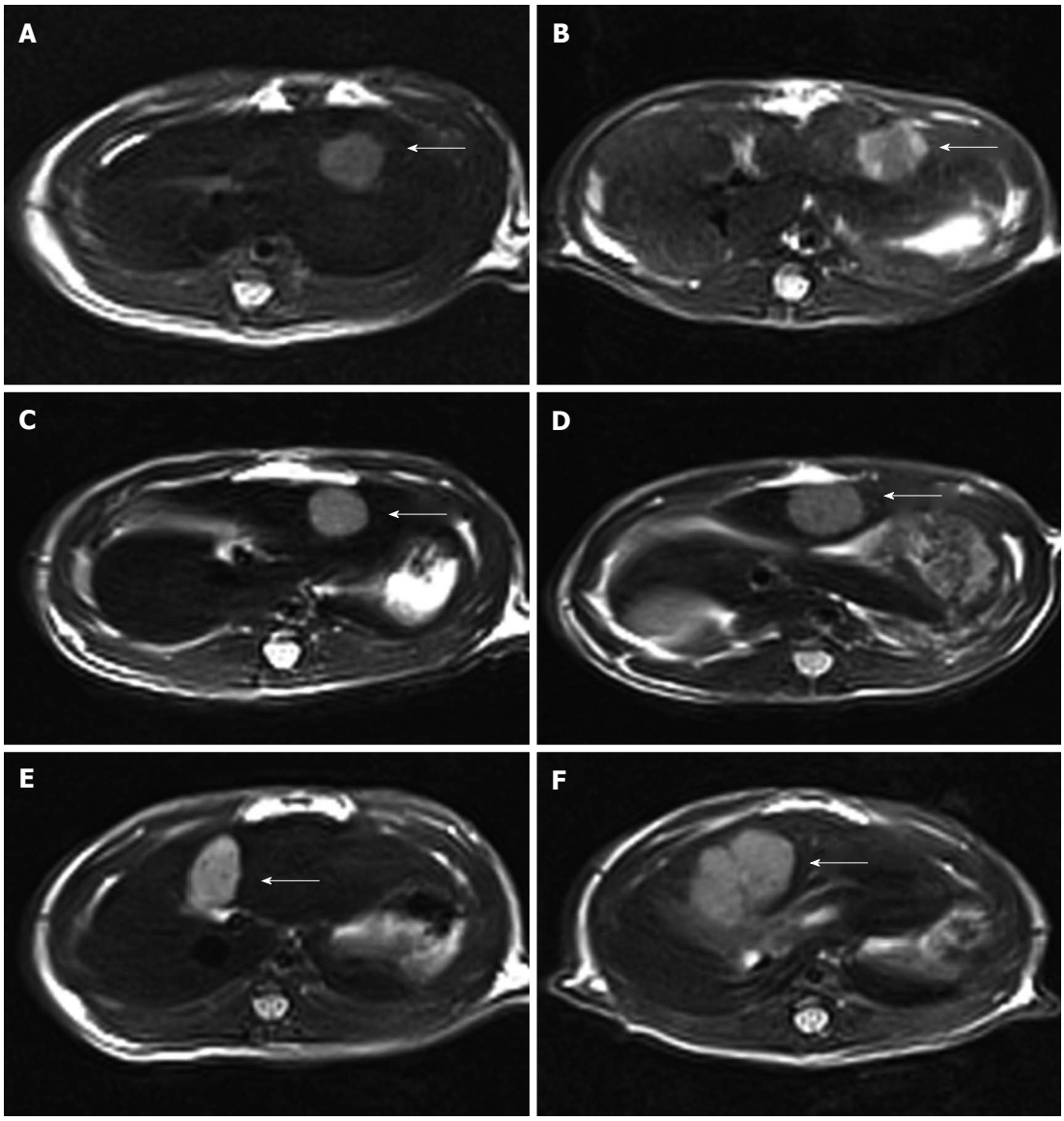

Figure 1 Representative transverse magnetic resonance images of solid liver tumors in group A (TACE+ GRGDSP loaded nanoparticles) (images A and B), group B (Control group 1, TACE+ GRGDSP) (images C and D) and group C (Control group 2, TACE alone) (images E and F) in ACI rats. 3870/80 matrix was acquired for all images. A: The pretreatment unenhanced T2-weighted TSE MR image shows a small hyperintense tumor (arrow) in the left lateral liver lobe (0.81 cm × 0.79 cm); B: On the posttreatment unenhanced T2-weighted TSE MR image, the same lesion (arrow) (0.85 cm × 0.82 cm) is a hyperintense tumor and has the inhomogeneous hypointense area corresponding to intratumoral necrosis. The growth of the hepatic tumor is noticeably inhibited after therapy; C: The pretreatment unenhanced T2-weighted TSE MR image shows a small hyperintense tumor (arrow) in the left lateral liver lobe (0.75 cm × 0.70 cm); D: On the posttreatment unenhanced T2-weighted TSE MR image, the same lesion (arrow) (1.03 cm × 0.87 cm) is a hyperintense tumor and has the inhomogeneous hypointense area corresponding to intratumoral necrosis. The hepatic tumor appears to have grown slightly after therapy; E: The pretreatment unenhanced T2-weighted TSE MR image shows a small hyperintense tumor (arrow) in the left lateral liver lobe (0.74 cm × 0.71 cm); F: On the posttreatment unenhanced T2-weighted TSE MR image, the same lesion appears as a 1.23 cm × 1.01 cm tumor (arrow) with relatively rapid growth compared to its size before therapy. TACE: Transarterial chemoembolization.

staining); 1 (0%-5%); 2 (6%-25%); 3 (26%-50%); 4 (51%-75%); and 5 (76%-100%).

Statistical analysis

The mean tumor growth ratio (V_2/V_1) by MRI from each group and the significance of differences were analyzed using the statistical software Prism (version 3.02, La Jolla, CA, United States).

Immunohistochemical staining of MMP-9 and VEGF was evaluated using descriptive and semiquantitative methods. Statistical analyses were performed using Prism (version 3.02, La Jolla, CA, United States).

Comparisons between groups were made using the Bonferroni test. Differences with a *P*-value less than 0.05 were considered statistically significant.

RESULTS

MRI examination

Tumor implantation was successful in all of the rats. Most tumors appeared homogeneous and were hypointense on T1-weighted images and hyperintense on T2-weighted images prior to treatment, but inhomogeneous after treatment. The mean growth

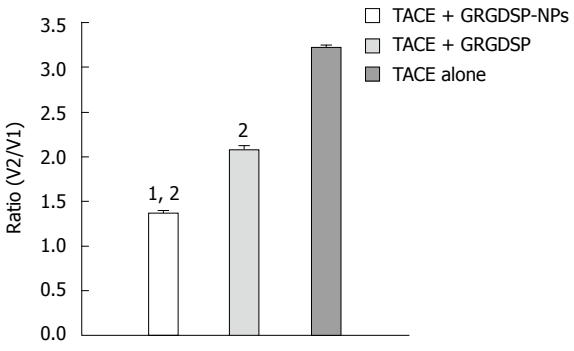

Figure 2 The mean tumor growth ratio (V_2/V_1) by magnetic resonance imaging from each group. The mean tumor growth ratio (V_2/V_1) by MRI showed significant differences between group A (TACE + GRGDSP loaded nanoparticles), group B (control group 1, TACE + GRGDSP) and group C (control group 2, TACE alone) ($P < 0.01$). TACE: Transarterial chemoembolization. [1]Compared with group B (control group 1, TACE + GRGDSP); [2]Compared with group C (control group 2, TACE alone).

ratios of tumors [V_2 (postreatment)/V_1 (pretreatment)] were 1.3649 ± 0.1194 in group A, 2.0770 ± 0.1595 in group B, and 3.2148 + 0.1075 in group C. Compared to groups B and C, group A (TACE + GRGDSP loaded nanoparticles) showed a significant reduction of tumor growth ($P < 0.01$) in the period of observation by Bonferroni test (Figures 1 and 2).

Immunohistochemical assay

The angiogenesis of tumor was evaluated using anti-VEGF antibody, and the metastasis of tumor was assessed using anti-MMP-9 antibody. MMP-9 and VEGF were expressed in all specimens. The immunoexpression of these proteins was confirmed by the presence of red cytoplasmic staining in tumor cells (Figures 3 and 4). Lower expression of MMP-9 and VEGF in HCC were observed in the group A than in groups B and C (controls) ($P < 0.01$) (Tables 1 and 2).

DISCUSSION

HCC is one of the most common malignancies with very high morbidity and mortality. TACE is a widely used palliative treatment for patients with unresectable HCC[4]. However, it has not led to significant improvements in the long-term survival rates, because of postoperative metastasis and recurrence of tumors[8]. Local infiltration and metastasis of tumors are a complicated process which is influenced by many factors.

The mechanism of adhesion molecules was reported to play an important role in the regulation of cellular migration, proliferation and apoptosis[17,18]. Integrin receptors are abnormally expressed on the surface of tumor cells, where they perform the basic function of mediating intercellular and cell-extracellular matrix (ECM) adherence. The adhesive function of integrins works by identifying the specific RGD sequence in the ligand (one part of ECM) and the links to it. RGD peptide is a kind of extrinsic peptide, which can competitively bind to integrin and inhibit binding with the RGD sequence of the ECM. The integrin-mediated adherence between tumor cells and ECM can be decreased by RGD peptide, and the inhibitory action is dose dependent. Furthermore, degradation of ECM caused by MMP-9 can be inhibited. Binding of RGD to integrin receptor $\alpha v\beta 3$, which is abnormally expressed in the endothelial cells of tumor blood vessels, may prevent blood vessel formation and infiltrating[17,19-21]. Therefore, RGD peptide can be regarded as a broad-spectrum antagonist of integrin. As a synthetic linear RGD peptide, GRGDSP (Gly-Arg-Gly-Asp-Ser-Pro) could inhibit the adherence of tumor cells to endothelial cells of blood vessels and limit its metastasis[22-24]. Tsuchiya *et al*[20] have found that intravenous administration of synthetic RGD pseudo-peptide (FC-336) could inhibit intrahepatic metastasis compared with control group ($P < 0.05$). Typically, a previous study has demonstrated that transarterial infusion of GRGDSP combined with TACE noticeably inhibited the growth of hepatic carcinoma and intrahepatic metastasis in Walker-256 rats[8]. Recently, nanobiotechnology has many advantages for improving drug delivery by the following approaches[10]. First, particle size can be reduced to nanometer size range to increase the surface area, thereby increasing the rate of dissolution. Second, nanoparticles can improve the absorption of insoluble compounds and macromolecules, enhance the bioavailability and release rates, and therefore reduce the amount of dose required and side effects. Finally, nanoparticles can be combined with ligands for targeted drug delivery. Nanotechnology is particularly useful for delivery of biological therapies. Nanotechnology will enable design and delivery of more effective drugs with increased efficacy and reduced toxicity. Wang *et al*[25] have showed that the nanoparticles coupled with RGD-peptide and doxorubicin represent high efficacy in inducing apoptosis in specific malignant cancer cells. Iwasaki *et al*[9] demonstrated that nanoparticles can be intravenously administrated for delivery of therapeutic genes with anti-tumor activity into human liver tumors. It was also reported that the therapeutic effect of adriamycin on liver malignancy can be significantly enhanced by its nanoparticle formulation and administration *via* the hepatic artery[11,12]. However, up to date no study has reported on the therapeutic effect of nanoparticles combined with TACE for treating HCC *in vivo* or in clinic. Thus, our experimental study was designed to reduce tumor progression and recurrence by combination of transarterial administration of GRGDSP loaded nanoparticles with TACE by using sandwich technique in an animal model of HCC. Our experimental results showed that transarterial administration of GRGDSP loaded nanoparticles + TACE can significantly inhibit the growth of hepatic tumor and intrahepatic metastases. Lower expression of MMP-9 and VEGF in HCC was observed in the group A (TACE + GRGDSP loaded-nanoparticles) than in group

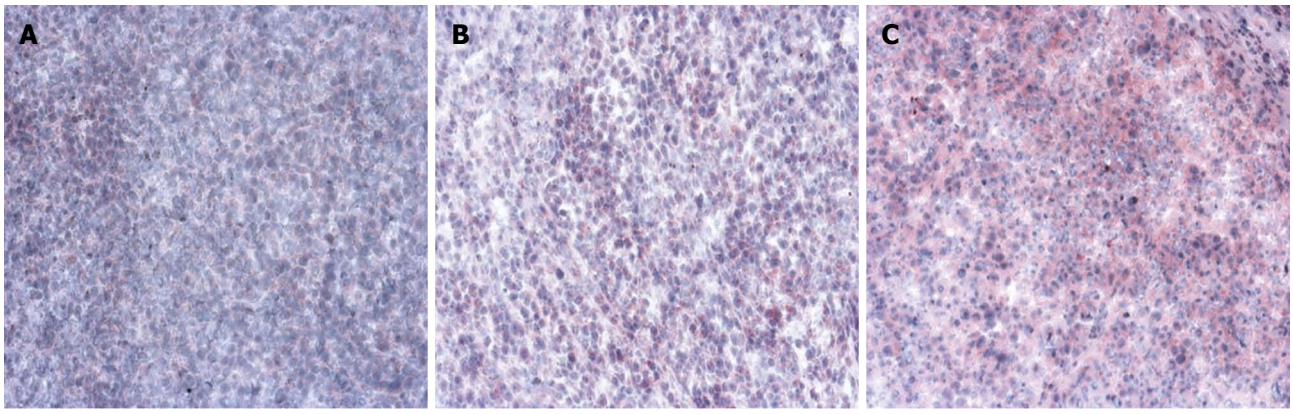

Figure 3 Immunohistochemical staining of VEGF in hepatocellular carcinoma in group A (TACE + GRGDSP loaded nanoparticles), group B (Control group 1, TACE + GRGDSP) and group C (Control group 2, TACE alone) (magnification × 100). A: VEGF expression in hepatocellular carcinoma in group A. B: Higher expression of VEGF in hepatocellular carcinoma was observed in group B than in group A; C: Higher immunohistochemical expression of VEGF in hepatocellular carcinoma was observed in group C than in groups A and B. VEGF: Vascular endothelial growth factor; TACE: Transarterial chemoembolization.

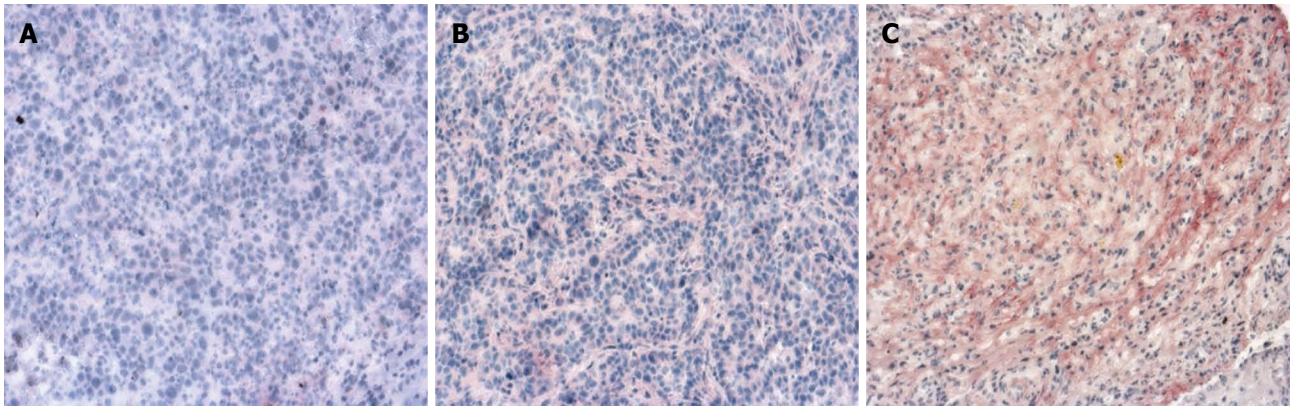

Figure 4 Immunohistochemical staining of matrix metalloprotein 9 in hepatocellular carcinoma in group A (TACE + GRGDSP loaded nanoparticles), group B (Control group 1, TACE + GRGDSP) and group C (Control group 2, TACE alone) (magnification × 100). A: Immunohistochemical staining of MMP-9 in hepatocellular carcinoma in group A. B: Higher immunohistochemical expression of MMP-9 in hepatocellular carcinoma was observed in group B than in group A. C: Higher immunohistochemical expression of MMP-9 in hepatocellular carcinoma was observed in group C than in groups A and B. MMP-9: Matrix metalloprotein 9; TACE: Transarterial chemoembolization.

Table 1 Immunohistochemical expression of matrix metalloprotein 9 and vascular endothelial growth factor in hepatocellular carcinoma (%) in groups A and B

	Group	TACE + GRGDSP						TACE + GRGDSP loaded nanoparticles						*P* value
	Score	0	1	2	3	4	5	0	1	2	3	4	5	
VEGF	Tumor	3	13	28	20	20	16	29	5	18	20	18	10	0.000
MMP-9	Tumor	0	1	17	18	42	22	0	6	36	41	12	5	0.000

MMP-9: Matrix metalloprotein 9; VEGF: Vascular endothelial growth factor; TACE: Transarterial chemoembolization.

B (TACE + GRGDSP) and group C (TACE alone). The invasive progression and metastases of tumor cells in group A were noticeably inhibited compared with the control groups.

For application in TACE, lipiodol not only occludes the small arteries supplying the tumor, but can also be used as the carriers bringing the anticancer drugs to the tumor. Lipiodol can deliver cytotoxic agents directly into tumor cells and endothelial cells, enter into the microcirculation of the tumor and block the blood flow[26-28]. Anticancer drugs administered through TACE can escape first-pass metabolism and have a prolonged half-life[29]. Moreover, the currently synthesized GRGDSP loaded nanoparticles have a mean diameter of 107 nm. It was documented that the passive targeting ability of the nanoparticles depends on the vessel microstructures of target organs. The nanoparticles with a diameter ranging from 20 to 300 nm have also the ability to directly enter the hepatocytes[30].

Table 2 Immunohistochemical expression of matrix metalloprotein 9 and vascular endothelial growth factor in hepatocellular carcinoma (%) in groups A and C

	Group	TACE						TACE + GRGDSP loaded nanoparticles						*P* value
	Score	0	1	2	3	4	5	0	1	2	3	4	5	
VEGF	Tumor	0	2	10	11	16	61	29	5	18	20	18	10	0.000
MMP-9	Tumor	0	6	4	0	9	81	0	6	36	41	12	5	0.000

MMP-9: Matrix metalloprotein 9; VEGF: Vascular endothelial growth factor; TACE: Transarterial chemoembolization.

In conclusion, encouraging results were obtained by combining transarterial administration of integrin inhibitor loaded nanoparticles with TACE for treating HCC in rats in comparison with control groups, and may prove valuable to human application as a therapeutic approach for the treatment of HCC. The combined multimodal targeting therapies reveal their enormous advantages as compared with conventional interventional therapy alone. However, detailed therapeutic mechanisms, therapeutic indications, monitoring and side effects of these combined therapies remain unclear and require more randomized experimental studies.

ACKNOWLEDGMENTS

The authors would like to thank all of those who participated in this study, and would like to thank professor Yan-Hong Zhu and Xiang-Liang Yang, The School of Life Science and Technology, Huazhong University of Science & Technology, Wuhan 430074, Hubei Province, China, for their selfless help to the preparation of the nanoparticles.

COMMENTS

Background

Transarterial chemoembolization (TACE) was introduced as one of the most common forms of interventional therapy but its therapeutic effect combined with gene therapy remains to be elucidated.

Research frontiers

Hepatocellular carcinoma (HCC) is one of the most commonly occurring tumors worldwide and TACE was introduced as an effective treatment in patients with unresectable HCC. Integrins expressed by tumor cells and host cells can contribute directly to the control and progress of metastatic dissemination. The authors have previously demonstrated the encouraging results of interventional therapy of TACE plus GRGDSP compared with TACE or GRGDSP alone. Transarterial infusion of integrin inhibitor (GRGDSP) loaded nanoparticles plus TACE may be a safe and effective therapy targeting metastatic dissemination of tumor cells.

Innovations and breakthroughs

This study for the first time evaluates the effects of TACE plus GRGDSP loaded nanoparticles compared with TACE alone or TACE plus GRGDSP for treating HCC in an animal model. Its results indicate that transarterial administration of GRGDSP loaded nanoparticles combined with TACE evidently retards tumor growth and intrahepatic metastases compared with TACE alone or TACE plus GRGDSP in rats.

Applications

Integrin inhibitor loaded nanoparticles combined with TACE might be used as a new therapeutic approach for the treatment of HCC and inhibition of intrahepatic metastasis after TACE.

Terminology

Integrin is a receptor molecule on the surface of cells, and its basic function is to mediate the intercellular adherence or adherence between cells and extracellular matrix. GRGDSP (Gly-Arg-Gly-Asp-Ser-Pro, integrin-inhibitor) can prevent the adhesion of tumor cells and endothelial cells of blood vessels, and also inhibit the metastasis of tumor cells.

Peer-review

The manuscript investigated the effect of combined administration of TACE and GRGDSP-conjugated nanoparticles in a rat model of HCC. The study appears to be well performed and the manuscript is well written. This study is more a pilot study rather than an exhaustive study, but is worth publishing. Subsequent studies should be performed to localize the nanoparticles shortly after the administration and to investigate the distribution of the nanoparticles in the liver.

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P- Reviewer: Piiper A **S- Editor**: Gong ZM **L- Editor**: Wang TQ
E- Editor: Zhang DN

World Journal of Gastroenterology

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DOI: 10.3748/wjg.v22.i21.5050

World J Gastroenterol 2016 June 7; 22(21): 5050-5059
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

ORIGINAL ARTICLE

Case Control Study

Danish cohort of monozygotic inflammatory bowel disease twins: Clinical characteristics and inflammatory activity

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Supported by Lundbeck foundation, Region of Southern Denmark, University of Southern Denmark, Hospital of Southern Jutland.

Institutional review board statement: The study was approved by the ethics committee of the region of southern Denmark (approval No: S20120176). Further, the study is included in the regional application to The Data Protection Agency (Institutional Southern Region of Denmark J.nr. 2008-58-0035). To ensure confidentiality direct paired comparisons between twin pairs are not shown.

Informed consent statement: Verbal as well as written informed consent was obtained from participants. This included consent to contact co-twins of the index twins, even if that included informing the co-twin of the diagnosis of the index twin.

Conflict-of-interest statement: Vibeke Andersen is an adviser for MSD/Merck, Jansen, and member of advisory board for MSD/Merck; Tine Jess has received funding for travel and speakers fee from AbbVie; Frederik Trier Moller, Lina Knudsen, Marcus Harbord, Jack Satsangi, Hannah Gordon, Lene Christiansen, Kaare Christensen: no conflicts of interest.

Data sharing statement: Technical appendix, code is available from the corresponding author at (frtm@ssi.dk). Additional data are available on request but may require further IRB approval/approvals from the data protection agency, to be shared outside the research group.

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Received: February 9, 2016
Peer-review started: February 10, 2016

First decision: March 7, 2016
Revised: March 21, 2016
Accepted: May 4, 2016
Article in press: May 4, 2016
Published online: June 7, 2016

Abstract

AIM: To describe the establishment of a Danish inflammatory bowel diseases (IBD) twin cohort with focus on concordance of treatment and inflammatory markers.

METHODS: We identified MZ twins, likely to be discordant or concordant for IBD, by merging information from the Danish Twin Register and the National Patient Register. The twins were asked to provide biological samples, questionnaires, and data access to patient files and public registries. Biological samples were collected *via* a mobile laboratory, which allowed for immediate centrifugation, fractionation, and storage of samples. The mean time from collection of samples to storage in the -80 ℃ mobile freezer was less than one hour. The diagnoses where validated using the Copenhagen diagnostic criteria.

RESULTS: We identified 159 MZ IBD twin pairs, in a total of 62 (39%) pairs both twins agreed to participate. Of the supposed 62 IBD pairs, the IBD diagnosis could be confirmed in 54 pairs. The cohort included 10 concordant pairs, whereof some were discordant for either treatment or surgery. The 10 concordant pairs, where both pairs suffered from IBD, included eight CD/CD pairs, one UC/UC pair and one UC/IBDU pair. The discordant pairs comprised 31 UC, 5 IBDU (IBD unclassified), and 8 CD discordant pairs. In the co-twins not affected by IBD, calprotectin was above 100 μg/g in 2 participants, and above 50 μg/g in a further 5 participants.

CONCLUSION: The presented IBD twin cohorts are an excellent resource for bioinformatics studies with proper adjustment for disease-associated exposures including medication and inflammatory activity in the co-twins.

Key words: Digestive system diseases; Inflammatory bowel diseases; Crohn's disease; Ulcerative colitis; Epidemiologic studies; Twins; Biobank

Core tip: Using co-twin study designs to segregate genetic and environmental factors in inflammatory bowel diseases (IBD) holds promise for future discovery, considering subclinical disease in the co-twins. However, as MZ IBD discordant twins are rarely seen this often-mean insufficient power for planned analyses. Hence, collaboration between IBD twin resources is crucial.

Moller FT, Knudsen L, Harbord M, Satsangi J, Gordon H, Christiansen L, Christensen K, Jess T, Andersen V. Danish cohort of monozygotic inflammatory bowel disease twins: Clinical characteristics and inflammatory activity. *World J Gastroenterol* 2016; 22(21): 5050-5059 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i21/5050.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i21.5050

INTRODUCTION

The inflammatory bowel diseases (IBD), Crohn's disease (CD) and ulcerative colitis (UC), affect a large number of Europeans[1,2]. Despite the introduction of new treatments, CD and UC remain chronic conditions with severe disease morbidity, often complicated by surgery and frequent admissions to hospital[1,3].

Although studies of the genome have found 200 loci associated with IBD, the variation in the IBD phenotype explained by these finding is still below 25%-30%[4,5] suggesting a role of environmental factors in IBD pathogenesis. Several studies indicate environmental impact on IBD pathogenesis including; exposure to pathogens[6], disease associated dysbiosis[7], metabolic disequilibrium[8], or epigenetic modifications[9]. More comprehensive studies, addressing these and other potential causes of IBD, could provide invaluable new insight into the pathogenesis of IBD[10], though studies using unrelated individuals would require large populations to overcome genetic variation between unrelated subjects[11].

Monozygotic (MZ) twins share common genotypes and epigenetic profiles at conception[12]. While some epigenetic differences arise during the lifetime of MZ twins[13], the inter-individual variation in relation to *e.g.,* the epigenome and the gut microbiome remain lower between twin pairs than between unrelated persons[14]. Consequently, comprehensive studies of the exposome using IBD discordant MZ twin study designs could prove a powerful tool to assess the combined effects of environmental and endogenous factors, and identify targets for treatment and prevention[15].

A major challenge in such discordant twin pair studies is that quiescent or subclinical disease may blur the boundaries between cases and their co-twin controls[16,17]. Another major challenge is that IBD discordant twin pairs are also treatment discordant, hence observed differences might derive from differential medication rather than disease discordance. Given enough power, studies using concordant twin pairs in addition to discordant twin pairs could allow researchers to adjust for disease-associated exposures such as medication, as both twins have IBD but may be discordant for some of the applied treatments. Further, calprotectin correlates with intestinal inflam-

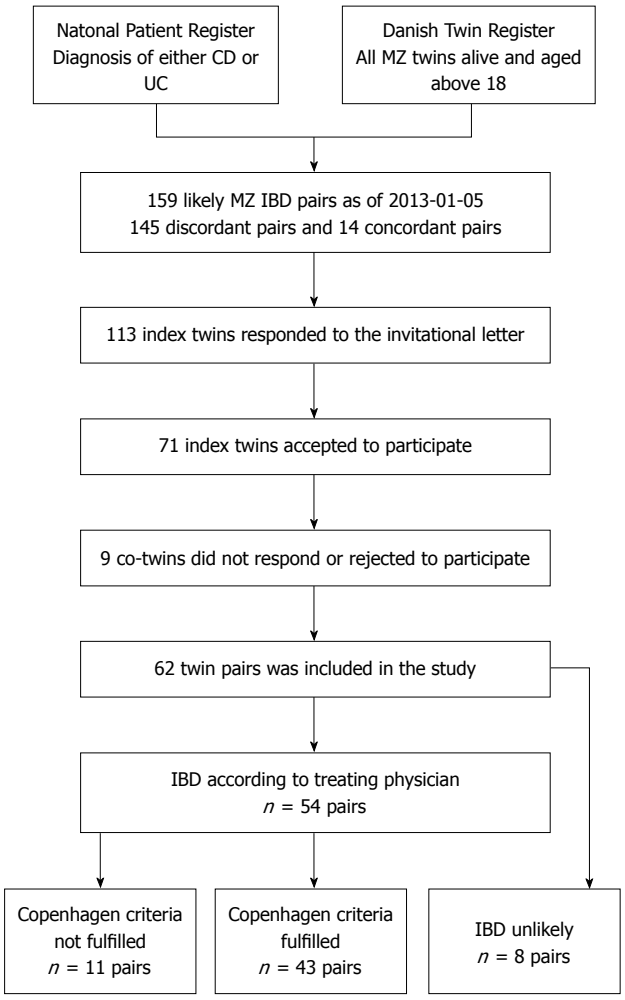

Figure 1 Collection of twin pairs. IBD: Inflammatory bowel diseases; CD: Crohn's disease; UC: Ulcerative colitis.

mation[18-20], and could reflect quiescent or subclinical disease in the unaffected co-twins.

We describe the establishment of a Danish IBD twin cohort including sampling of biological material, and illustrate the importance of treatment discordance and measurement of inflammatory markers for future bioinformatics studies using IBD affected twins.

MATERIALS AND METHODS

We identified MZ twins, likely to be discordant or concordant for IBD, by merging information from the Danish Twin Register and the National Patient Register[21,22].

Danish twin register

The Danish Twin Register enabled identification of MZ twins living in Denmark at the time of inclusion, with assessment of zygosity correct in 96% of cases[23]. The Register contains 72% of all twin pairs born between 1931-1968, with complete ascertainment of all live born twins since 1968[21].

National patient register

The National Patient Register is a nationwide register of all hospital discharge diagnoses, including surgical and other procedures recorded in Danish hospitals since 1977[22]. The Register provides outpatient data from 1994 and surgical procedures since 1996. Diagnoses of UC and CD were identified using the international classification of diseases (ICD) 8th and 10th revision codes for CD (563.00-563.09 and K50) and UC (563.19, 569.04 and K51).

The diagnosis of IBD has previously been found to be accurate in over 90% of IBD cases in the national patient register, using a pathology register as reference[24].

Cohort recruitment

Merging the Danish Twin Register and the National Patient Register, identified 159 MZ twin pairs in which at least one twin had a diagnosis of either CD or UC according to the National Patient Register as of May 1st 2013. Of these, 113 index twins (the first twin to contract IBD according to the register) responded to the invitational letter of whom 42 twins declined to participate. Of the 71 positive index twin responders, nine co-twins did not wish to participate, leaving 62 pairs for inclusion, Figure 1.

Data collection

The participants filled out a questionnaire including age, sex, smoking status, medication, dietary patterns including a food frequency questionnaire, a 48-h dietary recall, time of last meal or exercise, travel history, and pregnancies and disease activity at time of sampling, either Harvey Bradshaw Index (CD) (33) or Simple Clinical Colitis Index (UC) (34).

Data collected from the patient record included disease staging using the Montreal classification (32), any IBD complications, extra intestinal manifestations, and gastrointestinal operations as well as prior IBD medication.

The register diagnosis of CD, UC or IBDU, was validated by hospital records and pathology descriptions using the Copenhagen criteria[25]: Copenhagen Diagnostic Criteria for CD (at least two of the criteria present)[26,27]: (1) History of abdominal pain, weight loss and/or diarrhoea for more than three months; (2) Characteristic endoscopic findings of ulceration (aphthous lesions, snail track ulceration) or cobble stoning or radiological features of stricture or cobble stoning; (3) Histopathology consistent with Crohn's disease (epitheloid granuloma of Langerhans type or transmural discontinuous focal or patchy inflammation); and (4) Fistula and/or abscess in relation to affected bowel segments.

Copenhagen diagnostic criteria for UC (all three of the criteria present)[26,28]: (1) History of diarrhoea and/

Table 1 Clinical characteristics *n* (%)

Pair type	Discordant twin pairs					Concordant twin pairs	
Status	Co-twin	CD	IBDU	UC	non-IBD GI symptoms	IBD co-twin	IBD index twin
n	52	8	5	31	8	10	10
Males/Females	23/29	2/6	1/4	16/15	4/4	4/6	4/6
Age (yr)	50(26-78)	47 (26-67)	57 (34-77)	49 (32-70)	55 (27-78)	49 (28-68)	49 (28-68)
Age at onset		32 (21-47)	43 (23-73)	35 (20-59)	38 (23-62)	21 (14-29)	23 (11-34)
Age at diagnosis		34 (25-46)	41 (29-72)	34 (17-66)	48 (18-72)	24 (11-37)	31 (21-47)
CPH criteria fulfilled		6 (75)	4 (80)	24 (77)	0 (0)	8 (80)	9 (90)
Disease location							
L1 ileal		2 (25)	2 (40)			3 (30)	2 (20)
L2 colonic		3 (38)	0 (0)			1 (10)	1 (10)
L3 ileocolonic		0 (0)	0 (0)			4 (40)	4 (40)
L4 isolated upper disease		0 (0)	0 (0)			0 (0)	1 (10)
B1 non stricturing non penetrating		4 (50)	2 (40)			3 (30)	1 (10)
B2 stricturing		2 (25)	0 (0)			4 (40)	5 (50)
B3 penetrating		0 (0)	0 (0)			3 (30)	2 (20)
P perianal disease		1 (13)	0 (0)			2 (20)	0 (0)
Proctitis			1 (20)	6 (19)			
Left sided			0 (0)	9 (29)			
Extensive			1 (20)	10 (32)			

n denotes the number of participants with the phenotype described in status. IBD: Inflammatory bowel diseases; CD: Crohn's disease; UC: Ulcerative colitis.

or rectal bleeding and pus for more than one week or repeated episodes; (2) characteristic endoscopic findings of continuous ulceration, vulnerability or granulated mucosa; and (3) histopathology consistent with ulcerative colitis (neutrophils within epithelial structures, cryptitis, crypt distortion, crypt abscesses).

Inter-observer variation has previously been found with regards to the Montreal classification[29]. To avoid potential inter-observer variation one researcher validated the diagnoses and assessed the Montreal classification (FTM). Furthermore, to improve validity of diagnoses and phenotypes, complicated cases were reviewed by a gastroenterological specialist and senior physician (VAN). In daily clinical practice, the diagnosis may remain difficult; therefore, we included cases, which were perceived to have IBD by the treating physician although not fulfilling the Copenhagen criteria as IBD cases according to available information from the files. Cases where the diagnosis of IBD was unlikely were designated "Gastrointestinal (GI) symptoms not IBD" for future reference.

Biological samples

Due to the geographical challenges in sampling a nationwide cohort, a mobile lab was set up using a camper previously fitted for a similar purpose. The camper was equipped with a small lab bench, heating, refrigeration, -20 ℃ freezer, a mobile -80 ℃ freezer, as well as a swinging bucket centrifuge.

The mobile lab setup allowed researchers to visit the twins in their home or another private location. The samples were collected adhering to the Sample PRE-analytical Code (SPREC) and Biospecimen Reporting for Improved Study Quality (BRISQ) guidelines, logging primary container, pre- and post-centrifugation conditions, centrifugation parameters and storage conditions, see supplementary materials Table 1[30,31]. Faecal specimens were sampled by participants up until 48 h before the visit and stored in their own freezer at -20 ℃[32]. Samples were then transferred to a -80 ℃ freezer at the visit, under which conditions faecal samples have been found to be stable in composition[33]. Oral samples were collected with a cytobrush (Cytotak™ Transwab® Labelled Tube MW148) from the dorsum of the tongue, suspended in a buffer medium, and immediately frozen at -80 ℃. Paraffin was used to collect sputum samples that were either suspended in RNA later or frozen directly at -80 ℃. One researcher conducted the collection of all samples. All samples were analysed using standard methods centrally to avoid sampling variation between different centers.

The mean time from collection of samples to storage in the -80 ℃ mobile freezer was less than one hour, except for blood samples, which were 60 min and 15 s please see Supplementary materials Table 2. Records where kept to ensure identification of any deviations from protocol in future analysis.

Statistical analysis

The study included only basic descriptive statistics using R version 3.2.0. In order to ensure confidentiality, no grouping of the twins below five pairs was presented. The statistical methods of this study were reviewed by statistician Mikael Andersson from department of epidemiology at Statens Serum Institut.

RESULTS

Study cohort

Out of 62 MZ twin pairs, after scrutinizing patient records, register data, and questionnaires, we found the index case of eight pairs unlikely to have IBD.

Table 2 Complications, medication and smoking *n* (%)

Pair type	Discordant twin pairs					Concordant twin pairs	
Status	Co-twin	CD	IBDU	UC	non-IBD GI symptoms	IBD co-twin	IBD index twin
n	52	8	5	31	8	10	10
Complications							
GI complications[1]	3 (6)	2 (25)	1 (20)	2 (6)	0 (0)	4 (40)	5 (50)
Extra intestinal manifestations[2]	5 (10)	3 (38)	2 (40)	12 (39)	2 (25)	2 (20)	6 (60)
Ever surgery	0 (0)	2 (25)	1 (20)	12 (39)	2 (25)	7 (70)	5 (50)
Colectomy	0 (0)	1 (13)	1 (20)	7 (23)	0 (0)	0 (0)	0 (0)
Medication							
Ever TNF-inhibitor	0 (0)	2 (25)	1 (20)	4 (13)	0 (0)	1 (10)	1 (10)
Ever glucocorticoids	0 (0)	3 (38)	4 (80)	17 (55)	2 (25)	6 (60)	6 (60)
Ever other immunosupressor[3]	0 (0)	4 (50)	2 (40)	6 (19)	0 (0)	2 (20)	5 (50)
Ever 5-ASA	1 (2)	5 (63)	4 (80)	22 (71)	1 (13)	5 (50)	6 (60)
Smoking							
Never smoker	28 (54)	6 (75)	2 (40)	21 (68)	5 (63)	3 (30)	2 (20)
Current smoker	10 (19)	1 (13)	1 (20)	2 (6)	2 (25)	3 (30)	7 (70)
Former smoker	13 (25)	1 (13)	2 (40)	7 (23)	1 (13)	4 (40)	1 (10)

[1]Fistula, adherences, strictures, toxic megacolon, abscess, perforation, colorectal cancer; [2]Hepatitis, primary sclerosing cholangitis, autoimmune pancreatitis, uveitis, erythema nodosum, pyoderma gangrenosum, arthritis, aphthous ulcers; [3]Methotrexate, azathioprine, and cyclosporine;. *n* denotes the number of participants with the phenotype described in status. IBD: Inflammatory bowel diseases; CD: Crohn's disease; UC: Ulcerative colitis.

The 8 cases were afflicted by the following diagnoses: lymphocytic colitis, irritable bowel syndrome, *Clostridium Difficile* infection, ischemic bowel changes and abscesses without pathologic CD features and grouped as GI symptoms not IBD. At least one twin suffered from IBD in all remaining 54 pairs according to patient records before verification of diagnostic criteria. Forty-four were discordant for IBD, of whom 24 out of 31 UC pairs, four out of five IBDU pairs, and six out of eight CD pairs fulfilled the Copenhagen diagnostic criteria. Of the 10 concordant pairs, there were eight CD/CD pairs, one UC/UC pair, and one UC/IBDU pair, where all but one CD index twin fulfilled the Copenhagen criteria. Both verified and suspected cases were included in the cohort, to reflect clinical practice.

Age at diagnosis

The mean age at diagnosis was lower in the CD concordant than CD discordant pairs (24.75 years *vs* 31.75 years). The timespan between the diagnosis of an index twin and the IBD co-twin was 6 years on average, ranging from 94 d to 14 years. The mean disease duration at sampling was 15 years on average, ranging from 295 d to 37 years.

Clinical characteristics, complications, medication and smoking

Table 1 shows clinical characteristics of the discordant twin pairs. Nine extra intestinal manifestations were present among co-twins, most often arthropathy. Table 2 shows complications, medication and smoking. Though numbers are small, 25% of CD index twins and 63% of CD co-twins received surgery after their IBD diagnosis. Conversely, 50% received azathioprine among the CD index twins *vs* 13% among the CD co-twins.

Assessment of inflammatory activity in concordant and discordant twin pairs

Figure 2A shows inflammatory activity in discordant co-twin pairs as measured by calprotectin, at the time of sample collection. There was evidence of gut inflammation in the apparently non-affected co-twin, with faecal calprotectin > 100 μg/g in two individuals and > 50 μg/g in a further five (Figure 2A). In two of the index twins whose IBD diagnosis could not be verified, faecal calprotectin was > 100 μg/g. Values of patient reported disease scores were also slightly increased though slightly less pronounced (Figure 2B and C).

DISCUSSION

We have established a nationwide cohort of 62 affected or suspected IBD monozygotic twin pairs, which allow assessment of a range of disease- and treatment-associated and phenotypical traits amongst both discordant and concordant MZ IBD twins. Validation of the CD, UC, and IBDU diagnoses resulted in 8 pairs where the diagnosis was unlikely, and 11 pairs where the diagnosis was likely, but the clinical information was too sparse to validate this. Therefore, 43 twin pairs fulfilled the Copenhagen diagnostic criteria. The cohort included 10 concordant pairs, and several of these IBD concordant pairs were discordant for either treatment or surgery. The 44 IBD discordant pairs comprised 31 UC pairs, five IBDU pairs, and eight CD pairs. Inflammatory activity was above the normal range in 7 of the co-twins not affected by IBD, with calprotectin above 100 μg/g in two co-twin pairs and above 50 μg/g in a further five pairs.

The strength of the presented twin cohort lies in the wide range of data collected, from questionnaire

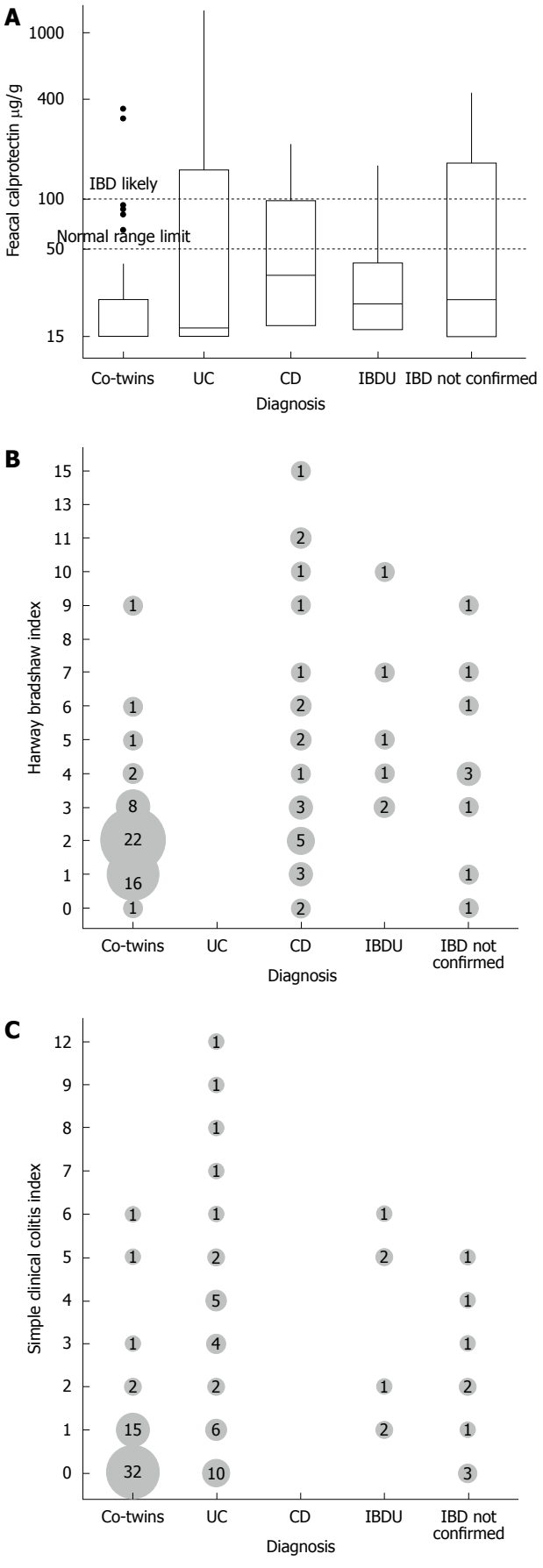

Figure 2 Figure shows fecal calprotectin measures stratified (A), harwey bradshaw index stratified by phenotype (B) and simple clinical colitis index stratified (C) by phenotype. IBD: Inflammatory bowel diseases; CD: Crohn's disease; UC: Ulcerative colitis.

data, patient file and public register data, to multiple biological samples. Our mobile laboratory enabled uniform collection of biological material with few deviations from existing guidelines regarding sample collection, storage, and handling. The average time from sampling to storage at -80 ℃ was 1 h or less for all samples. Our uniform sample collection method using a mobile laboratory reduced aberrant data handling normally affecting nationwide multicentre studies, and allowed for a single-site analysis of disease activity data. A drawback of this approach is that more advanced laboratory handling, like cell separation and preserving viable cells, was not performed. Instead, the study used CPT tubes, a commercial cell preservative, and gradual freezing of cells using a "mister frosty", which has previously been shown to preserve viable cells and cell integrity[34].

While the collection of biological material in this study is more uniform than previous twin studies[14,35-40], we were unable to perform invasive manoeuvres such as endoscopy with our mobile setup. Though we expect to achieve access to some biopsy material taken from routine endoscopies, a large proportion of the healthy twins had not recently undergone endoscopy, thus limiting opportunities for comparison. Our assessment of clinical characteristics and IBD medication use aggregated data from patient files and questionnaire data. Consequently, treatment not documented by hospital-based physicians or recalled by patients may remain unaccounted for, but this potential bias should be similar between concordant and discordant pairs.

Our inclusion rate was lower than expected at 62 pairs out of the contacted 159, perhaps due to the extent of collected samples, and the need for including both twins. Indeed, some selection bias favouring the inclusion of concordant pairs over discordant pairs could not be ruled out, based on the proportion of concordant pairs invited to the proportion of concordant pairs accepting to participate.

The IBD twins were identified using nationwide registers, reducing bias often bestowed upon twin studies relying on advertising for recruitment. Given sufficient power, concordant pairs may play a crucial role in discerning the effects of disease-associated traits, such as medical therapy, from the effects of IBD, *e.g.,* on the methylome or metagenome. In addition, though we did not have the power to test this formally, the mean age at diagnosis seemed lower in the CD concordant pairs at 25 years *vs* 32 years among CD discordant pairs. Results from previous twin studies are conflicting on this point[41-43]. If indeed such a difference exists, one possible explanation might be that concordant pairs carry a larger genetic liability to disease, with a lower threshold for disease throughout life, increasing the risk of both twins contracting IBD, resulting in twin concordance. A previous Swedish twin study found the total allele frequency of *Nod/Card* mutations to be 4.4 times higher among concordant twin pairs compared to discordant twin pairs contributing to, but not explaining concordance[41].

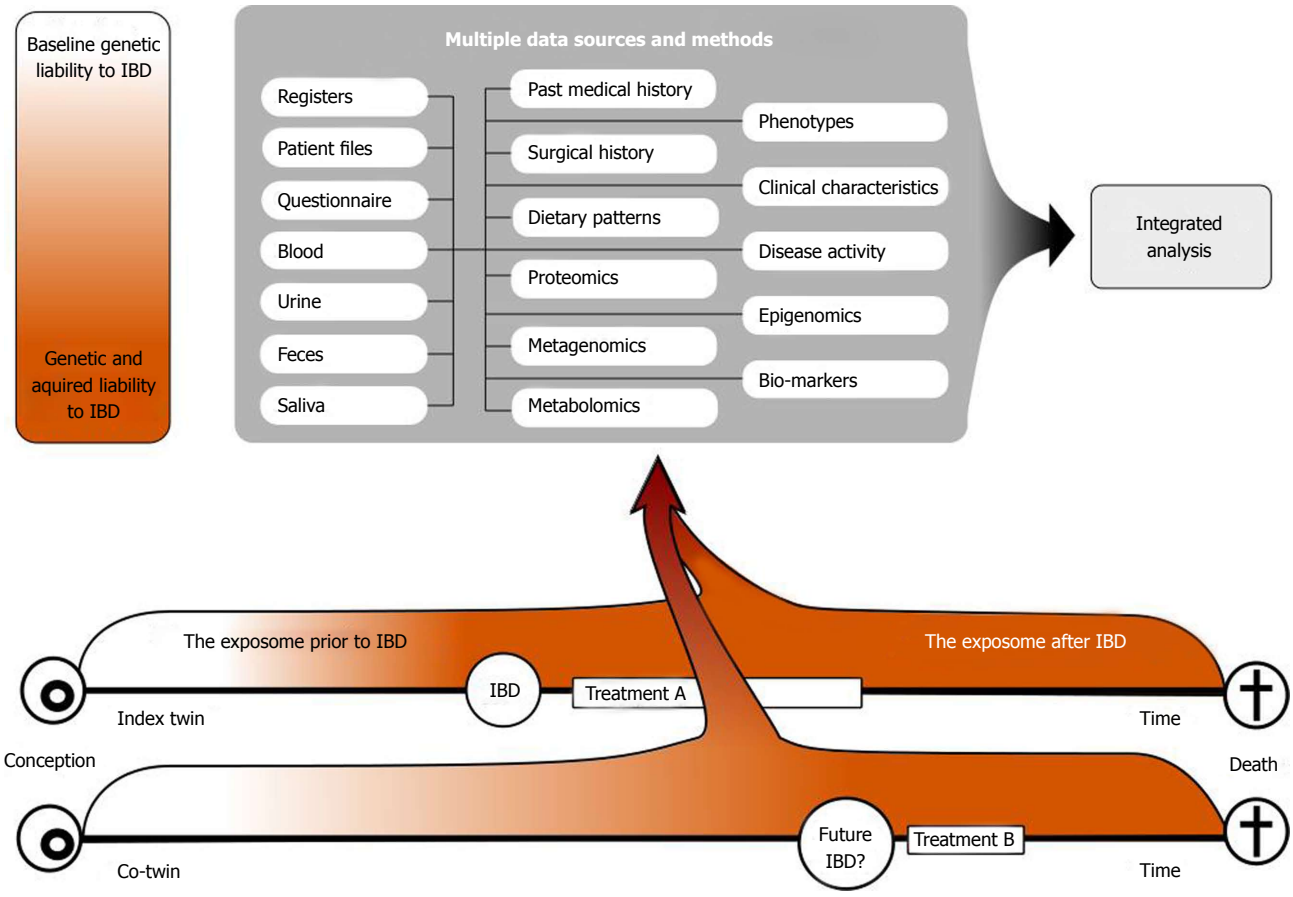

Figure 3 Figure shows how the collected twin data may be used in different downstream analyses. The figure illustrates the initial genetic concordance in liability, the progressive discordance for disease liability due to heterogeneous exposures, and the possible future concordance for IBD, but not necessarily for treatment. IBD: Inflammatory bowel diseases.

Phenotypical characteristics differentiate CD and UC from each other and from other conditions such as IBS, microscopic colitis and infections. A combination of clinical evaluation, endoscopic, histological, radiological, and/or bio-chemical investigations provides the diagnostic foundation for CD and UC[44,45]. The correct classification of twin discordance is paramount. Of note, classification is not only dependent on the correct diagnosis but also on time interval following the diagnosis of the index twin, with the risk of contracting IBD declining with time for the co-twin. Although the maximum time-span between concordant pairs in this cohort was below the mean disease duration of 15 years in the present cohort, final verification of twin discordance can only be assessed at the end of the lifespan of both twins. Methods do however exist that take time to event into account[46]. While future disease may not be a problem in a discordant twin study design, this is only true if the exposures causing this disease are not already present. Quiescent and subclinical disease may complicate the distinction between cases and their co-twin controls. Indeed, a newly published study indicates the presence of latent or emerging disease in family members of affected IBD cases[16]. In addition, family members of affected IBD cases have increased calprotectin levels as compared to the background population[17]. Though a normal calprotectin level does not exclude IBD, due to the dynamic nature of this condition, and a low level in well treated IBD patients, calprotectin levels correlate with intestinal inflammation[18-20], and could thus reflect an increased liability to IBD in familial members of IBD-affected cases[47,48]. One[48] twin study published in the past 10 years has reported on increased levels of intestinal inflammatory activity biomarkers such as calprotectin among the unaffected co-twins, while the majority of previous twin studies have not[14,35-40,49]. We found two co-twins with no history of IBD with calprotectin values exceeding 100 μg/g, and a further 5 with values above the normal range. This may be important, as increased calprotectin may reflect that many of the exposures leading to disease may already be present in a co-twin, if subclinical disease is not already present. As a result, inter-individual differences with impact on disease pathogenesis within pairs may be harder to assess, suggesting that calprotectin levels should be considered in analysis.

Disease discordant IBD twins remain rare and precious to research[14,35-40,49]. Though providing a powerful model for research, this will often mean insufficient power for planned analyses. Hence, collaboration between twin resources is crucial. Collaboration with

the Nixon Twin and Multiplex (TAM) United Kingdom IBD cohort, analysing epigenetic data within similar biological material, has already been established[50]. Thus, both the Danish and the British IBD twin cohorts will include a range of clinical, epidemiological and biological data enabling researchers to study a cross section of the IBD exposome (Figure 3).

The present cohort demonstrates the importance of assessing inflammatory biomarkers reflecting subclinical inflammatory activity among otherwise healthy co-twins in discordant twin studies. The present cohort will be part of international collaborations, thereby increasing the power to detect disease-associated factors, and allow sufficient concordant twins to be included in studies to adjust for treatment effects. Hypotheses that may be tested include whether epigenetic differences controlling IBD loci previously identified in GWAS studies exists within the twin pairs. Other approaches may include rodent models where rodent responses to biological material from discordant pairs may differ. Consequently, analysis of a range of data from cohorts of monozygotic IBD pairs using bioinformatic methods such as metagenomics, metabolomics, proteomic and epigenetics could provide new insight into the role of the exposome in IBD pathogenesis.

COMMENTS

Background

Although studies of the genome have found 200 loci associated with inflammatory bowel diseases (IBD), the variation in the IBD phenotype explained by these finding is still below 25%-30%, suggesting a role of environmental factors in IBD pathogenesis. Several studies indicate environmental impact on IBD pathogenesis, including exposure to pathogens, disease-associated dysbiosis, metabolic disequilibrium, or epigenetic modifications. Comprehensive studies of the exposome using IBD discordant MZ twin co-twin study designs could prove a powerful tool to assess the combined effects of environmental and endogenous factors, and identify targets for treatment and prevention.

Research frontiers

Historically twin studies have been used to calculate the heritability of complex traits and diseases. The co-twin control design constitutes an excellent model to investigate environmental factors associated with disease due to the genetic match between monozygotic twins. To date only a few studies have applied this method using bioinformatics methods in IBD. Most prominent is the work of Jonas Halvfosen and his group in Orebro Sweden that identified differential microbial stool patterns between IBD discordant twin pairs, underlining the potential of this methodology.

Innovations and breakthroughs

Co-twin control designs may result in complexity reduction, thus increasing power to identify microbial or epigenetic patterns associated with IBD and the interplay between these complex traits. Such studies necessitate cohorts as the one described in this study designed for downstream bioinformatics studies, and special emphasis was on pre-analytical sample handling.

Applications

The present cohort demonstrates the importance of assessing inflammatory biomarkers reflecting subclinical inflammatory activity among otherwise healthy co-twins in discordant twin studies. Using co-twin study designs to investigate environmental determinants of disease holds promise for future discovery. However, as MZ IBD discordant twins are rare this often means insufficient statistical power. Hence, collaboration between twin resources is crucial. Through international collaborations analysis of a range of data from cohorts of monozygotic IBD pairs using bioinformatic methods such as metagenomics, metabolomics, proteomics and epigenetics could provide new insight into the role of the exposome in IBD pathogenesis.

Terminology

Concordant twin pairs: twin pairs where both twins are affected by disease or trait. Discordant twin pairs: twin pairs where only one twin is affected by disease or trait. According to Wild (2005), the exposome encompasses all human environmental exposures from conception onwards.

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As the authors realize, the real strength of this cohort is in the future translational studies, primarily as it relates to epigenetics. While they very briefly and superficially discuss these plans in the last paragraph of the conclusion, expanding on future plans for hypothesis-driven translational research would further strengthen the manuscript. Otherwise, this is a nice introduction to a novel cohort that hopes to generate fascinating future work.

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P- Reviewer: Shapiro JM **S- Editor**: Qi Y **L- Editor**: A
E- Editor: Ma S

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DOI: 10.3748/wjg.v22.i21.5060

World J Gastroenterol 2016 June 7; 22(21): 5060-5067
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

ORIGINAL ARTICLE

Case Control Study

Serum *Helicobacter pylori* KatA and AhpC antibodies as novel biomarkers for gastric cancer

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Author contributions: Tian WJ and Zhao YS designed research; Fan Q and Ren XY collected and collated the data; Zhang B, Li HL, Guo F, Zhou HB and Zhu JW performed the assays; Zhang B and Tian WJ analyzed the data; and Zhang B wrote the paper.

Supported by the National Natural Science Foundation of China, No. 81573219; and Heilongjiang Province Office of Education Foundation, No. 12541288.

Institutional review board statement: The study was reviewed and approved by the Committee of Human Research of Harbin Medical University, Harbin, China.

Informed consent statement: All study participants provided informed written consent prior to study enrollment.

Conflict-of-interest statement: The authors declare that there is no conflict of interest related to this study.

Data sharing statement: No additional data are available.

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Received: December 24, 2015
Peer-review started: December 25, 2015
First decision: January 13, 2016
Revised: January 27, 2016
Accepted: February 22, 2016
Article in press: February 22, 2016
Published online: June 7, 2016

Abstract

AIM: To investigate catalase (KatA) and alkyl hydroperoxide reductase (AhpC) antibodies of *Helicobacter pylori* as biomarkers for gastric cancer (GC).

METHODS: This study included 232 cases and 264 controls. Recombinant KatA and AhpC proteins were constructed and the levels of antibodies were tested by indirect enzyme-linked immunosorbent assay (ELISA). Logistic regression was applied to analyze the relationships between KatA, AhpC and GC. The χ^2 trend test was used to evaluate the dose-response relationships between serum KatA and AhpC antibody levels and GC. Receiver operating characteristic (ROC) curve was used to evaluate the screening accuracy of KatA and AhpC as biomarkers. Combined analysis was used to observe screening accuracy of predictors for GC.

RESULTS: In all subjects, the association between KatA and AhpC and GC risk was significant ($P < 0.001$) with odds ratio (OR) = 12.84 (95%CI: 7.79-21.15)

and OR = 2.4 (95%CI: 1.55-3.73), respectively. KatA and AhpC antibody levels were strongly related to GC risk with a dose-dependent effect (*P* for trend < 0.001). The area under the ROC (AUC) for KatA was 0.806, providing a sensitivity of 66.81% and specificity of 86.36%; and the AUC for AhpC was 0.615, with a sensitivity of 75.65% and specificity of 45.49%. The AUC was 0.906 for KatA and flagella protein A (FlaA) combined analysis.

CONCLUSION: Serum KatA and AhpC antibodies are associated with GC risk and KatA may serve as a biomarker for GC. KatA/FlaA combined analysis improved screening accuracy.

Key words: *Helicobacter pylori*; Catalase; Serum antibody; Alkyl hydroperoxide reductase; Gastric cancer; Case-control study

Core tip: Effective screening methods for gastric cancer (GC) have remained limited to date. The aim of this study was to explore whether serum catalase and alkyl hydroperoxide reductase antibodies of *Helicobacter pylori* could serve as novel and reliable biomarkers for GC monitoring.

Zhang B, Li HL, Fan Q, Guo F, Ren XY, Zhou HB, Zhu JW, Zhao YS, Tian WJ. Serum *Helicobacter pylori* KatA and AhpC antibodies as novel biomarkers for gastric cancer. *World J Gastroenterol* 2016; 22(21): 5060-5067 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i21/5060.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i21.5060

INTRODUCTION

Gastric cancer (GC) is the fifth most common malignancy and the third leading cause of cancer-related death worldwide[1]. Although the overall incidence rate of GC continues to fall, there were still almost 1 million new cases of GC in 2012[2]. *Helicobacter pylori (H. pylori)* are micro-aerophilic gram-negative bacteria that cause inflammatory reactions by selectively colonizing the gastric mucosa. The International Agency for Research on Cancer has classified *H. pylori* as a category I carcinogen since 1994[3]. Epidemiological data also support that *H. pylori* infection is strongly associated with GC[4-6], increasing risk by up to six-fold[7]. In contrast, increasing data shows that *H. pylori* eradication significantly decreases the development of GC[8,9], particularly in high-risk populations with no precancerous lesions[10]. Eradication of *H. pylori* seems a reasonable approach for preventing GC. However, nearly 50% of the population worldwide is infected with *H. pylori*[11]. Mass eradication therapy in the general population may bring about development of antibiotic-resistant strains of *H. pylori* as well as over-consumption of medical resources. Therefore, there is an urgent and need to identify a reliable screening biomarker for GC.

It is reported that only a small fraction of patients infected with *H. pylori* have severe clinical outcomes, such as gastric ulcer (10%), atrophic gastritis (5%), and gastric malignancy (2%)[3]. Research indicates that these different outcomes may be associated with the virulence factors of *H. pylori*[12-14]. Catalase (KatA) and alkyl hydroperoxide reductase (AhpC) virulence factors play a crucial role in protecting *H. pylori* from oxidative stress and maintaining a stable environment for the growth of bacteria[15,16]. Huang *et al*[17] confirmed that KatA and AhpC were over expressed under the condition of oxidation stress (H_2O_2) in *H. pylori* strains isolated from patients with GC, gastritis, or duodenal ulcer. We previously reported that serum flagella protein A (FlaA) antibody of *H. pylori* may serve as noninvasive biomarker for early detection of GC[18]. In this study, combined analysis was applied to explore the screening value of KatA, AhpC, and FlaA for GC. This study aims to assess the correlations between KatA and AhpC and GC and explore whether they could serve as novel and reliable biomarkers for GC.

MATERIALS AND METHODS

Study subjects

This was a hospital-based case-control study, which was approved by the Committee of Human Research of Harbin Medical University, Harbin, China. Two hundred and thirty-two cases of GC were primarily diagnosed by pathology at the Third Affiliated Hospital of Harbin Medical University between April and July 2010. The controls comprised 182 healthy people chosen from the Harbin Xiangfang Center for Disease Control and Prevention and 82 cancer-free people recruited from the neurology department at the Fourth Affiliated Hospital of Harbin Medical University between March and July 2011. All participants gave signed informed consent, and we completed a face-to-face questionnaire that included age, sex, smoking status, and alcohol consumption. Venous blood samples of 5 mL were collected from all participants, centrifuged at 3000 r/min, and stored at -80 ℃.

Cloning and expression of recombination protein

A clinical strain of *H. pylori* provisionally named H015a was isolated from a GC patient at the Second Affiliated Hospital of Harbin Medical University. Genomic DNA of H015a was extracted as a template using a DNA extraction kit (QIAGEN, Valencia, CA, United States). The *katA* and *ahpC* gene coding sequences were obtained from Genbank. Amplification of *katA* and *ahpC* gene fragments was implemented by polymerase chain reaction (PCR). The PCR primers were designed using Primer Premier 5.0 software. For *katA*, the primer sequences were 5'-CCGGAATTCATGGTTAATAAAGATGTGAACA-3'

(forward) and 5′-CCGCTCGAGTTACTTTTTCTTTTT-TGTGTGG-3′ (reverse) that generated a 1518 bp fragment. For *ahpC*, the primer sequences were 5′-CCGGAATTCATGTTAGTTACAAAACTTGCCC-3′ (forward) and 5′-CCGCTCGAGTTAAAGCTTAATG-GAATTTTC-3′ (reverse) that generated a 597 bp fragment. *Eco*RI and *Xho*I restriction endonuclease sites were incorporated into the forward and reverse primer sequences of these two genes, respectively. Amplification was implemented under the following conditions: 30 cycles of 94 ℃ for 30 s, 55 ℃ for 30 s, and 72 ℃ for 90 s followed by a final extension at 72 ℃ for 7 min. Subsequently, two PCR products were cloned into the cloning vector pMD18-T and transformed into *Escherichia coli* (*E. coli*) strain DH5α. The positive clones were screened and cloned into the prokaryotic expression vector pET-32a. The recombinant plasmids *katA*-pET-32a and *ahpC*-pET-32a were introduced into *E. coli* BL21 (DE3) cells for expression of recombinant proteins, respectively. The target sequences of *katA* and *ahpC* gene were assayed by the dideoxy chain termination method (Biotechnology firm, BGI, Beijing, China). The recombinant *katA*-pET-32a-BL21 and *ahpC*-pET-32a-BL21 strains were cultured in lysogeny broth (LB) with 100 μg/mL ampicillin, and induced at 30 ℃ by isopropylthio-β-d-galactoside with a final concentration of 1 mmol/L and 0.5 mmol/L, respectively. *E. coli* cells were harvested after 4 h and disrupted ultrasonically. The suspension and precipitate were collected and protein expression was analyzed by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

H. pylori serological tests

A serological test for *H. pylori* immunoglobulin (Ig)G antibodies has already been completed and described by our group[17].

Purification and renaturation of target recombinant proteins

The recombinant proteins were purified by Ni-NTA His Bind resin (Novagen, Darmstadt, Germany). We used stepwise dialysis to obtain the fusion protein by removing the denaturant (urea) in the purified protein. The dialysis tube was boiled 10 min in buffer (2% $NaHCO_3$ and 1mmol/L EDTA pH 8.0) and EDTA solution (1 mmol/L) sequentially. After cooling, the purified protein was put into the dialysis tube, and both ends were clamped with the dialysis clips. The protein was dialyzed in urea solution (pH 8.3) with a slowly decreasing concentration: 6 mol/L, 4 mol/L, 2 mol/L, 1 mol/L and 0 mol/L. Each dialysis lasted 24 h. Finally, the sample was removed from the dialysis tube and stored at -80 ℃ until analysis.

Detection of antibodies against recombinant proteins with enzyme-linked immunosorbent assay

An indirect enzyme-linked immunosorbent assay (ELISA) was applied to detect the serum antibodies against *H. pylori* recombinant KatA and AhpC proteins. Recombinant KatA and AhpC proteins were diluted to 2 μg/mL and 0.25 μg/mL, respectively. Proteins at 100 μL/well were incubated in a 96-well micro-plate (Costar, Washington, DC, United States) at 4 ℃ overnight and washed three times with phosphate buffer saline, Tween-20 (PBST), followed by blocking with 10% goat serum (AR0009; Boster, Beijing, China) and incubation for 2 h at 37 ℃. Serum sample from cases and controls diluted 3200-fold with 10% bovine serum albumin (BSA) was added to the plate at 100 μL/well and incubated for 1 h at 37 ℃. Each serum sample was tested in three parallel wells. The plate was again washed three times with PBST. Peroxidase-conjugated goat anti-human IgG (H+L) (ZSGB-Bio, Beijing, China) was diluted 1:5000 with buffer, and 100 μL was added to each well and incubated 30 min at 37 ℃. Tetramethylbenzidene (TMB) substrate buffer was added to the plate at 100 μL/well and incubated in the dark for 15 min at 37 ℃, Fifty microliters stop solution was added per well to terminate the reaction. Finally, the plate was read at 450 nm absorbance using a micro-plate reader (Biotech Synergy 2, Winooski, Vermont, United States). The determination of serostatus of antibody was based on optical density (OD) value. The optimal cutoff point of OD values was used to classify samples as seropositive or seronegative.

Statistical analysis

All statistical analyses were conducted using SPSS 22.0 version software (Armonk, NY, United States). Unconditional logistic regression analysis was performed to estimate odds ratio (OR) and 95% confidence interval (CI) for the relationship between GC and antibodies. The χ^2 trend test was used to assess dose-response relationships between serum KatA and AhpC antibody levels and GC. In addition, a receiver operating characteristic (ROC) curve was plotted to identify the cutoff point of serum KatA and AhpC antibody results. Sensitivity, specificity, and area under the ROC curve (AUC) with 95%CI were calculated to evaluate the screening value of serum KatA and AhpC antibody levels for GC. Moreover, the optimal cutoff value was determined by the maximum Youden index (Youden index = sensitivity + specificity - 1). Combined analysis was used to observe screening accuracy of predictors for GC. For all tests, $P < 0.05$ was considered statistically significant.

RESULTS

Characteristics of study subjects

The characteristics of the study subjects were described in our previous study[18].

Cloning and expression of the recombinant proteins

Nucleotide homology of the cloned *katA* gene compared to *H. pylori 26695* was 95.52% The homology of

Table 1 Association between gastric cancer and seropositivity of catalase and alkyl hydroperoxide reductase antibodies in study subjects *n* (%)

Virulence factors serostatus	All subjects				*H. pylori* positive subjects				*H. pylori* negative subjects			
	Case	Control	OR (95%CI)	*P* value[1]	Case	Control	OR (95%CI)	*P* value[1]	Case	Control	OR (95%CI)	*P* value[1]
KatA												
Negative	78 (33.62)	228 (86.36)	1.0 (Reference)	< 0.001	47 (35.61)	104 (88.14)	1.0 (Reference)	< 0.001	26 (29.21)	109 (83.85)	1.0 (Reference)	< 0.001
Positive	154 (66.38)	36 (13.64)	12.84 (7.80-21.15)		85 (64.39)	14 (11.86)	14.59 (6.84-31.13)		63 (70.79)	21 (16.15)	12.15 (5.79-25.51)	
AhpC												
Negative	56 (24.14)	121 (45.83)	1.0 (Reference)	< 0.001	33 (25.00)	57 (48.31)	1.0 (Reference)	< 0.001	54 (54.00)	103 (70.55)	1.0 (Reference)	< 0.001
Positive	176 (75.86)	143 (54.17)	2.40 (1.55-3.73)		99 (75.00)	61 (51.69)	2.30 (1.25-4.23)		46 (46.00)	43 (29.45)	2.04 (1.10-3.78)	
Combination of KatA and AhpC												
Negative	78 (33.62)	226 (85.61)	1.0 (Reference)	< 0.001	49 (37.12)	104 (88.14)	1.0 (Reference)	< 0.001	33 (33.00)	127 (86.99)	1.0 (Reference)	< 0.001
Positive	154 (66.38)	38 (14.39)	11.64 (7.12-19.01)		83 (62.88)	14 (11.86)	13.40 (6.29-28.53)		67 (67.00)	19 (13.01)	13.91 (6.74-28.74)	

[1]The *P* value was obtained from logistic regression analysis adjusted for age, sex, family history of gastric cancer, smoking, and alcohol consumption. KatA: Catalase; AhpC: Alkyl hydroperoxide reductase.

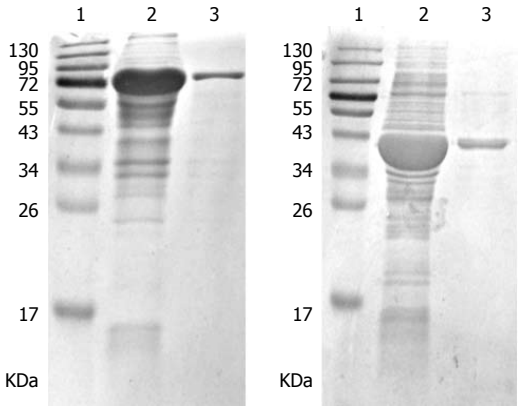

Figure 1 SDS-PAGE analysis of purified recombinant proteins. A: KatA; B: AhpC. 1: Marker; 2: Unpurified protein; 3: Purified protein. KatA: Catalase; AhpC: Alkyl hydroperoxide reductase.

ahpC nucleotide was 96.48% compared with *H. pylori J99.*

A prokaryotic expression system was constructed. After induction by isopropyl beta D thiogalactoside (IPTG), proteins with the expected size were clearly present as inclusion bodies in the ultrasonic precipitation by SDS-PAGE. Finally, the purified fusion proteins were obtained (Figure 1).

Association between serum positivity of antibodies and GC

As shown in Table 1, an association between KatA and GC risk was observed, with OR = 12.84 (95%CI: 7.79-21.15), 14.59 (6.84-31.13), and 12.15 (5.79-25.51) in all, *H. pylori*-positive and *H. pylori*-negative subjects, respectively ($P < 0.001$). Dose-dependent effects showed that KatA antibody levels were strongly related to GC risk in the three populations mentioned above (P for trend < 0.001) (Table 2). Similarly, a significant association between GC risk and serum positivity of AhpC was observed with OR = 2.40 (95%CI: 1.55-3.73) in all subjects, 2.30 (1.25-4.23) in *H. pylori*-positive subjects, and 2.04 (1.10-3.78) in *H. pylori*-negative subjects ($P < 0.001$) (Table 1). Correspondingly, AhpC antibody level was significantly related to GC risk in a dose-dependent manner (P for trend < 0.001) (Table 2). Moreover, an evident association between GC risk and serum positivity of combination of KatA and AhpC was present, with OR = 11.64 (95%CI: 7.12-19.01), 13.39 (6.29-28.53), and 13.91 (6.74-28.74) in all, *H. pylori*-positive and *H. pylori*-negative subjects, respectively ($P < 0.001$) (Table 1).

Screening utility of serum antibody for GC

An ROC curve was plotted to explore the screening value of KatA and AhpC for GC. The AUC for KatA was 0.806 (95%CI: 0.768-0.845), 0.805 (0.751-0.853), and 0.801 (0.741-0.861) in all, *H. pylori*-positive and *H. pylori*-negative subjects, respectively (Figure 2). The AUC for AhpC was 0.615 (95%CI: 0.566-0.665) in all subjects, 0.629 (0.560-0.699) in *H. pylori*-positive subjects, and 0.605 (0.530-0.680) in *H. pylori*-negative subjects (Figure 3). As shown in Table 3, the optimal cutoff value of KatA and AhpC for GC was 0.3583 and 0.3647 in all subjects, providing a sensitivity of 66.81% and 75.65% and a specificity of 86.36% and 45.49%, respectively. AUC for the combination of KatA and FlaA was 0.906 (95%CI: 0.879-0.932), and the optimal cutoff value was 0.4305 with a sensitivity of 78.88% and a specificity of 89.02% (Figure 4A). For combination of KatA, FlaA, and AhpC, the AUC was 0.910

Table 2 Dose-dependent association between gastric cancer risk and serum catalase and alkyl hydroperoxide antibodies levels in study subjects *n* (%)

All subjects					*H. pylori* positive subjects					*H. pylori* negative subjects				
Antibody level (OD)[1]	Case	Control	OR (95%CI)[2]	*P* value for trend	Antibody level (OD)	Case	Control	OR (95%CI)[2]	*P* value for trend	Antibody level (OD)	Case	Control	OR (95%CI)[2]	*P* value for trend
KatA														
≤ 0.4187	167 (71.98)	66 (25.0)	1.0 (Reference)	< 0.001	≤ 0.4152	92 (69.70)	29 (24.58)	1.0 (Reference)	< 0.001	≤ 0.4167	66 (74.16)	32 (24.58)	1.0 (Reference)	< 0.001
0.4187-0.5313	36 (15.52)	66 (25.0)	4.25 (2.49-7.27)		0.4152-0.5133	23 (17.42)	30 (25.42)	3.79 (1.78-8.06)		0.4167-0.5568	9 (10.11)	33 (25.42)	6.67 (2.70-16.51)	
0.5313-0.6799	18 (7.76)	66 (25.0)	9.95 (5.05-19.62)		0.5133-0.6692	9 (6.82)	30 (25.42)	9.69 (3.81-24.70)		0.5568-0.6824	11 (21.36)	33 (25.42)	7.00 (2.68-18.30)	
> 0.6799	11 (4.74)	66 (25.0)	15.85 (6.97-36.06)		> 0.6692	8 (6.06)	29 (24.58)	16.55 (5.51-49.76)		> 0.6824	3 (3.39)	32 (24.58)	19.89 (4.32-91.70)	
AhpC														
≤ 0.2168	88 (37.93)	66 (25.00)	1.0 (Reference)	< 0.001	≤ 0.2182	51 (38.64)	29 (24.58)	1.0 (Reference)	< 0.001	≤ 0.2110	31 (34.83)	32 (24.58)	1.0 (Reference)	< 0.001
0.2168-0.3265	69 (29.74)	66 (25.00)	1.26 (0.76-2.11)		0.2182-0.3433	41 (31.06)	30 (25.42)	1.10 (0.54-2.25)		0.2110-0.3310	29 (32.58)	33 (25.42)	1.44 (0.69-3.00)	
0.3265-0.4888	49 (21.12)	66 (25.00)	1.41 (0.82-2.43)		0.3433-0.4908	28 (21.21)	30 (25.42)	1.54 (0.70-3.38)		0.3310-0.4948	16 (17.98)	33 (25.42)	1.83 (0.80-4.23)	
> 0.4888	26 (11.21)	66 (25.00)	3.54 (1.84-6.82)		> 0.4908	12 (9.09)	29 (24.58)	3.40 (1.32-8.73)		> 0.4948	13 (14.61)	32 (24.58)	3.33 (1.31-8.46)	

[1]Serum positivity for the antibodies to KatA and AhpC was categorized by quartiles of antibody levels in controls; [2]Adjusted for age, sex, family history of gastric cancer, smoking, and alcohol consumption. *H. pylori*: *Helicobacter pylori*; KatA: Catalase; AhpC: Alkyl hydroperoxide reductase.

(95%CI: 0.885-0.935), offering a sensitivity of 80.17% and a specificity of 88.64%, while the optimal cutoff value was 0.4354 (Figure 4B).

DISCUSSION

Gastric carcinogenesis is a multifactorial process, and *H. pylori* infection plays an important role in the initial stage[19]. Patients with malignant tumors are often diagnosed at an advanced stage, and 5-year survival rate is < 10%[20]. Therefore, early detection is a crucial factor for GC prevention. However, it is difficult to diagnose GC any earlier because the symptoms of gastric pre-cancerous and malignant diseases are non-specific and vague. At present, endoscopy is the gold standard for screening GC and is commonly used in the clinic. A large case-control study from Japan indicated that GC mortality was reduced 30% by endoscopic screening compared with no screening[21]. In spite of this finding, limitations of endoscopy, such as the existence of over diagnosis and unwillingness of asymptomatic patients because of pain as well as cost make endoscopy unsuitable for population-based screening. Serological testing is widely available and is a low-cost noninvasive diagnostic method. In the present study, we explored whether serum *H. pylori* antibody could serve as a biomarker for GC monitoring.

KatA is a ubiquitous enzyme that protects *H. pylori* cells from extracellular H_2O_2 attack[22,23] and plays an important role in colonization of gastric mucosa[15]. AhpC is the most abundant and essential antioxidant protein of *H. pylori*[16], and it protects bacteria from lipid peroxidation and DNA damage[24,25]. We used a commercial ELISA method to detect *H. pylori* infection status. However, this method may fail to detect prior *H. pylori* infection in GC patients, and patients positive for anti-CagA (cytotoxin-associated gene A) antibody may have negative results for *H. pylori* serological testing[26,27]. In order to eliminate these possible influences on our results, *H. pylori*-negative and overall subjects were also analyzed to observe the associations between GC and the KatA and AhpC antibodies. The results indicated that we should be more vigilant regarding antibody titer and seropositivity. Meanwhile, we found that the median of KatA and AhpC antibody levels were lower in cases group than in the controls (data not shown). This finding implied that the high antibody titer of *H. pylori* KatA and AhpC may protect against the occurrence of GC.

A Latin American study showed that seropositivity of KatA in a population within a high risk of GC area was higher than that in a low-risk population[28]. Our results confirmed that KatA was associated with GC, and seropositivity of KatA antibody showed a 14.59-fold increased risk of GC. Yan *et al*[29] found that AhpC antibody of *H. pylori* may be related to the development of gastric diseases using the gerbil model to simulate human *H. pylori* infection. In addition, Huang *et al*[30] indicated that AhpC was expressed in greater amounts in GC than gastritis strains. In our study, there was a significant association between AhpC antibody and GC, based on epidemiology data. Further analysis found that KatA and AhpC antibody levels were strongly related to GC risk in a dose-dependent manner. In order to explore whether KatA and

Table 3 Sensitivity and specificity of different catalase and alkyl hydroperoxide reductase critical values

Percentile[1]	All subjects			*H. pylori* positive subjects			*H. pylori* negative subjects		
	Critical value (OD)[2]	Sensitivity	Specificity	Critical value (OD)[2]	Sensitivity	Specificity	Critical value (OD)[2]	Sensitivity	Specificity
KatA									
Optimal cutoff point[2]	0.3583	66.81%	86.36%	0.3557	64.39%	88.14%	0.3730	70.79%	83.85%
25%	0.2800	46.55%	93.18%	0.4152	69.70%	74.58%	0.2773	50.56%	90.00%
50%	0.4305	75.00%	71.59%	0.5133	87.12%	49.15%	0.4447	78.65%	69.23%
75%	0.5958	90.95%	36.36%	0.6692	93.94%	24.58%	0.6107	92.13%	36.15%
90%	0.7418	97.84%	16.29%	0.9042	100.00%	8.47%	0.7873	97.75%	14.62%
AhpC									
Optimal cutoff point[2]	0.3647	75.65%	45.49%	0.3613	75.76%	48.31%	0.2330	43.82%	70.77%
25%	0.1953	30.43%	78.95%	0.1953	30.30%	80.51%	0.1917	30.34%	77.69%
50%	0.2830	59.57%	57.14%	0.2865	59.85%	60.17%	0.2913	62.92%	57.69%
75%	0.4267	84.35%	32.71%	0.4325	83.33%	33.05%	0.4313	85.39%	23.85%
90%	0.5747	95.65%	14.29%	0.5302	93.94%	13.56%	0.6410	96.63%	13.85%

[1]Percentiles of serum KatA and AhpC antibody levels in controls; [2]Optimal cutoff point in the different parameters was identified according to the maximum Youden's index (sensitivity + specificity - 1). *H. pylori*: *Helicobacter pylori*; KatA: Catalase; AhpC: Alkyl hydroperoxide reductase.

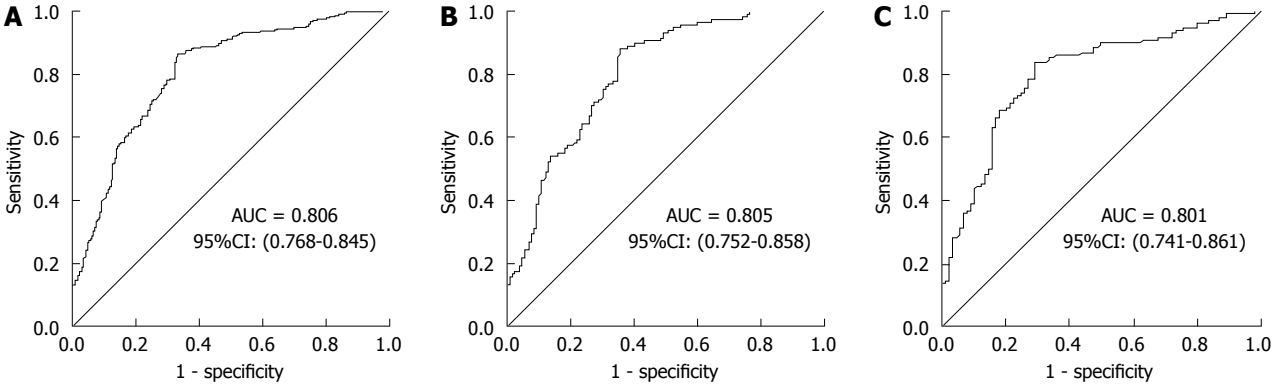

Figure 2 Receiver operating characteristic curve for serum catalase antibody. A: All subjects; B: *H. pylori*-positive subjects; C: *H. pylori*-negative subjects. *H. pylori*: *Helicobacter pylori*.

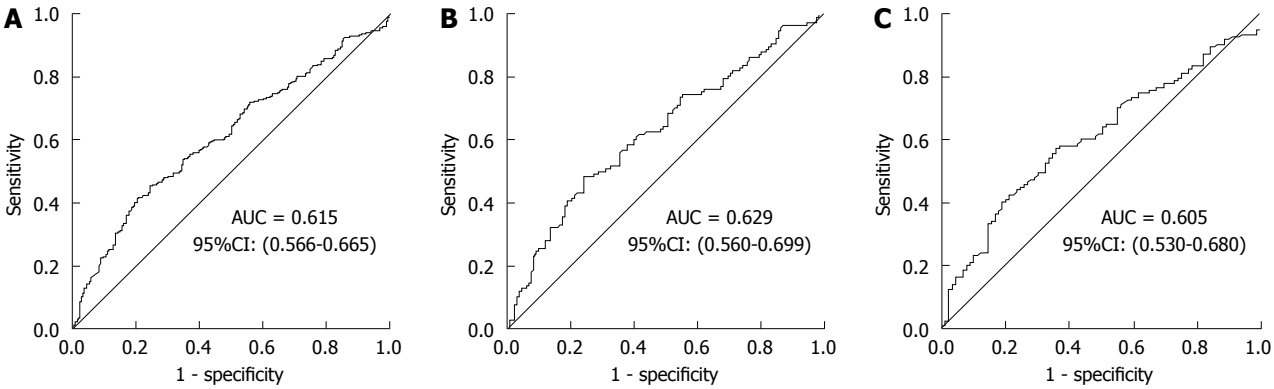

Figure 3 Receiver operating characteristic curve for serum alkyl hydroperoxide reductase antibody. A: All subjects; B: *H. pylori*-positive subjects; C: *H. pylori*-negative subjects. *H. pylori*: *Helicobacter pylori*.

AhpC could serve as biomarkers for GC, ROC curves were plotted to evaluate the screening value of the antibodies. The results showed that the AUC for KatA was 0.806, which was higher than the general standard for diagnosis (AUC $\geqslant$ 0.7)[31,32]. Unfortunately, the AUC for AhpC was lower. Generally, a single indicator for screening has a lower screening yield. At this point, we attempted to develop a combined analysis to assess the value of screening. Our previous study found that the sensitivity was 74.1%, and the specificity was 64.4%, while FlaA served as a screening biomarker for GC alone[17]. The combined results for KatA, FlaA, and AhpC showed that the AUC for combination of KatA and FlaA was elevated by 0.10, and sensitivity and specificity

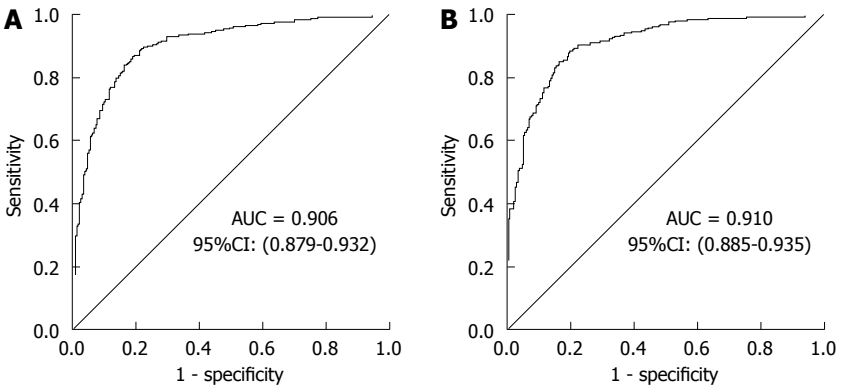

Figure 4 Receiver operating characteristic curve for combined analysis in all subjects. A: KatA + FlaA; B: KatA + FlaA + AhpC. KatA: Catalase; AhpC: Alkyl hydroperoxide reductase; FlaA: Flagella protein A.

were increased by 12.07% and 2.66%, respectively, in all subjects compared to KatA alone. Yet, combination of KatA, FlaA, and AhpC did not improve screening power in the identification of patients with GC compared to combination of KatA and FlaA.

Indirect ELISA method was adopted to detect serum KatA and AhpC antibodies in this study, and this method might be accompanied by the non-specific signal caused by cross-reactivity. In other words, KatA and AhpC will not only react with the corresponding specific antibody but also with the non-specific antibodies in the present study, Because of this non-specific signal, some *H. pylori*-negative subjects were classified as KatA or AhpC positive.

Some evidence indicates that *H. pylori* infection increases the risk of non-cardia GC[7,33]. Nine (3.88%) cardia GC cases were included in our study. However, their involvement did not affect the overall results and conclusion.

In conclusion, the data indicate that serum KatA and AhpC antibodies are associated with GC risk and that KatA may serve as a novel biomarker for GC screening. Combined analysis of KatA and FlaA could improve screening accuracy. However, serum AhpC antibody performed poorly as a marker for GC. Our study offers a basis for early diagnosis of GC, and further prospective studies are needed to verify our findings.

COMMENTS

Background

Helicobacter pylori (*H. pylori*) infection is a crucial cause of gastric cancer (GC). Eradication of *H. pylori* seems a reasonable approach for preventing GC, but it is not feasible in large populations due to financial limitations. Therefore, a sensitive and low-cost screening biomarker for GC is urgently needed.

Research frontiers

Invasive endoscopy is the gold standard for GC detection, but it is not suitable for population-based screening. Serological testing is a widely available and noninvasive diagnostic method. In this study, the authors explored the value of serum catalase (KatA) and alkyl hydroperoxide reductase (AhpC) antibodies of *H. pylori* as biomarkers for GC monitoring.

Innovations and breakthroughs

This study indicated that KatA and AhpC antibodies are associated with GC risk and that KatA may serve as a novel biomarker for GC screening. Besides, combining for KatA and flagella protein A could improve screening accuracy.

Applications

These finding offers a basis for early diagnosis of GC.

Peer-review

This is a well-designed study showing that KatA and AhpC antibodies are associated with GC. The methodology is well described. Exploration of KatA and AhpC as biomarkers has important value for GC prevention.

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P- Reviewer: Aoyagi K, Vorobjova T **S- Editor**: Ma YJ
L- Editor: Filipodia **E- Editor**: Wang CH

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DOI: 10.3748/wjg.v22.i21.5068

World J Gastroenterol 2016 June 7; 22(21): 5068-5078
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

ORIGINAL ARTICLE

Retrospective Cohort Study

Endoscopy-based management decreases the risk of postoperative recurrences in Crohn's disease

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Author contributions: All the authors equally contributed to this paper.

Institutional review board statement: The study was approved by local Ethics Committee (IRB number 00008526 - 2014/CE86).

Informed consent statement: The study was performed in accordance with the Declaration of Helsinki, Good Clinical Practice and applicable regulatory requirements.

Conflict-of-interest statement: All the authors have no conflict of interest related to the manuscript.

Data sharing statement: The original anonymous dataset is available on request from the corresponding author at a_buisson@hotmail.fr.

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Received: February 24, 2016
Peer-review started: February 26, 2016
First decision: March 31, 2016
Revised: April 14, 2016
Accepted: April 20, 2016
Article in press: April 20, 2016
Published online: June 7, 2016

Abstract

AIM: To investigate whether an endoscopy-based management could prevent the long-term risk of postoperative recurrence.

METHODS: From the pathology department database, we retrospectively retrieved the data of all the patients operated on for Crohn's disease (CD) in our center (1986-2015). Endoscopy-based management was defined as systematic postoperative colonoscopy (median time after surgery = 9.5 mo) in patients with

no clinical postoperative recurrence at the time of endoscopy.

RESULTS: From 205 patients who underwent surgery, 161 patients (follow-up > 6 mo) were included. Endoscopic postoperative recurrence occurred in 67.6%, 79.7%, and 95.5% of the patients, respectively 5, 10 and 20 years after surgery. The rate of clinical postoperative recurrence was 61.4%, 75.9%, and 92.5% at 5, 10 and 20 years, respectively. The rate of surgical postoperative recurrence was 19.0%, 38.9% and 64.7%, respectively, 5, 10 and 20 years after surgery. In multivariate analysis, previous intestinal resection, prior exposure to anti-TNF therapy before surgery, and fistulizing phenotype (B3) were postoperative risk factors. Previous perianal abscess/fistula (other perianal lesions excluded), were predictive of only symptomatic recurrence. In multivariate analysis, an endoscopy-based management (n = 49/161) prevented clinical (HR = 0.4, 95%CI: 0.25-0.66, P < 0.001) and surgical postoperative recurrence (HR = 0.30, 95%CI: 0.13-0.70, P = 0.006).

CONCLUSION: Endoscopy-based management should be recommended in all CD patients within the first year after surgery as it highly decreases the long-term risk of clinical recurrence and reoperation.

Key words: Crohn's disease; Postoperative recurrence; Endoscopy; Prevalence; Risk factors

Core tip: Although often recommended, the impact of an endoscopy-based management following surgery remains poorly investigated in Crohn's patients. We aimed to investigate whether an endoscopy-based management could prevent the long-term risk of postoperative recurrence in Crohn's disease (CD). We retrospectively retrieved the data of 161 patients operated on for CD in our center. We showed for the first time, that an endoscopy-based management decreased the long-term risk of clinical and surgical postoperative recurrence in CD and the risk of reoperation.

Boucher AL, Pereira B, Decousus S, Goutte M, Goutorbe F, Dubois A, Gagniere J, Borderon C, Joubert J, Pezet D, Dapoigny M, Déchelotte PJ, Bommelaer G, Buisson A. Endoscopy-based management decreases the risk of postoperative recurrences in Crohn's disease. *World J Gastroenterol* 2016; 22(21): 5068-5078 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i21/5068.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i21.5068

INTRODUCTION

Crohn's disease (CD) is a chronic inflammatory bowel disease (IBD) of unknown etiology and can lead to digestive damage[1,2]. In the era of biologics, surgery still remains required in half of the patients ten years after diagnosis, especially in complicated diseases i.e. stenosis, abscess or fistula[3]. Surgical resection is unfortunately not curative in CD, and postoperative recurrence (POR) remains a crucial issue in these patients. The risk of reoperation is very heterogeneous in the medical literature due to different studies periods and designs, but ranges from 12% to 57% 10 years after surgery[4-7]. While clinical POR occurred in approximately half of the patients 10 years after surgery[8], three quarters (48%-93%) of patients experienced endoscopic POR within one year after surgery in referral centers[8-20].

More than 25 years ago, Rutgeerts *et al*[12] underlined that postoperative history of CD is very heterogeneous and highlighted the need to identify predictive factors of recurrence to stratify CD patients in order to optimize the therapeutic management in the immediate postoperative period. Several factors have been proposed as POR predictors (smoking, perianal lesions, previous intestinal resection, fistulizing phenotype and resection length > 50 cm), but their impact remains still debated[8,21].

Performing an endoscopy within the first year after surgery is often recommended in clinical practice[21,22]. However, the level of evidence suggesting the efficacy of such strategy remains low. Two retrospective studies reported no impact of an endoscopy-based management (EBM)[23,24]. A French group suggested, in a retrospective cohort, that an EBM was associated with a decrease risk of clinical POR at 5 years[25]. Recently, the landmark POCER trial showed that an early EBM decreased the risk of endoscopic POR at 18 mo post-surgery[26]. However the long-term impact of EBM on the risks of clinical and surgical POR remains unknown.

In the present study, we aimed to investigate whether an EBM could prevent the long-term risk of POR in CD. In addition, we aimed to report the prevalence and the risk factors of endoscopic, clinical and surgical POR, in our cohort, between 1986 and 2015.

MATERIALS AND METHODS

Ethical considerations

The study was performed in accordance with the Declaration of Helsinki, Good Clinical Practice and applicable regulatory requirements. The study was approved by local Ethics Committee (IRB number 00008526 - 2014/CE86).

Patients

We performed a retrospective study of a single-center cohort in which standardized evaluation was completed by experienced clinicians in all patients.

Table 1 Baseline characteristics of the 161 included Crohn's disease patients at the time of surgery *n* (%)

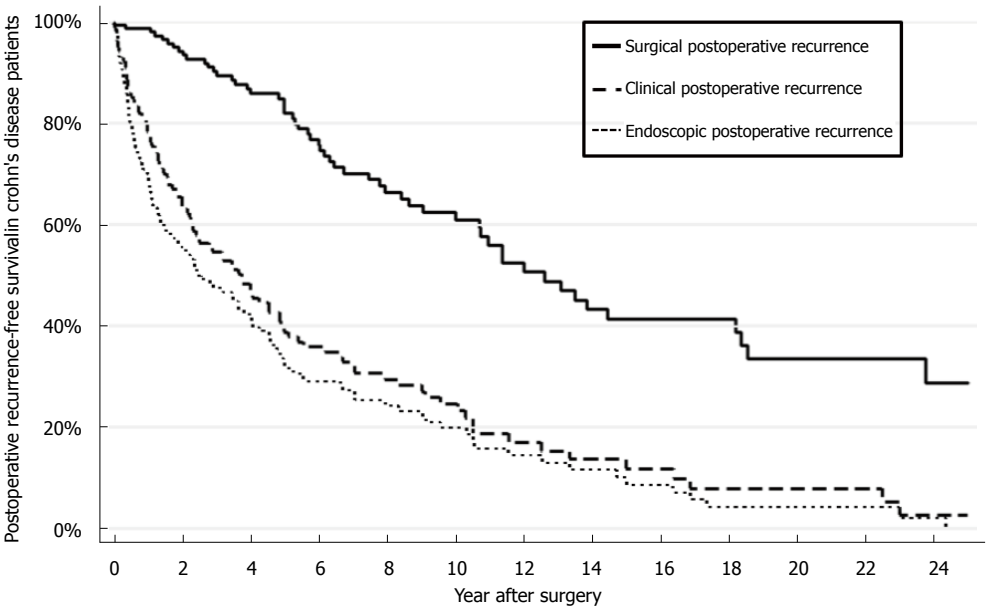
Mean age at the time of surgery (yr)	36.4 ± 13.4	Adalimumab	22 (13.7)
Mean age at diagnosis (yr)	28.7 ± 13.1	Anti-TNF naive at the time of surgery	37 (23.0)
Median disease duration (yr) (IQR)	5.8 (2.0–11.7)	Type of surgery	
Female gender	93 (57.8)	Ileocecal resection	76 (47.2)
Mean weight (kg)	60.2 ± 14.8	Ileal resection	21 (13.1)
Mean body mass index (kg/m²)	21.5 ± 4.9	Ileo-colectomy	14 (8.7)
Active smoker	53 (32.9)	Partial colectomy	31 (19.2)
Familial history of IBD	20 (12.4)	Subtotal colectomy	8 (5.0)
Previous appendectomy	67 (41.6)	Total colectomy	9 (5.6)
Previous intestinal resection	50 (31.1)	Abdomino-perianal amputation	2 (1.2)
Montreal classification		Site of anastomosis	
Age at diagnosis		Ileo-colic	91 (66.4)
A1	15 (9.3)	Ileo-rectal	9 (6.6)
A2	116 (72.1)	Ileo-ileal	21 (15.3)
A3	28 (17.4)	Colo-colonic	31 (22.6)
Crohn's disease location		Colo-rectal	7 (5.1)
L1	64 (39.8)	Stomia	
L2	21 (13.0)	None	113 (70.2)
L3	75 (46.6)	Transitory	39 (24.2)
L4	18 (11.2)	Definitive	9 (5.6)
Crohn's disease behaviour		Surgical technic of anastomosis	
B1	12 (7.4)	Stapled	46 (43.8)
B2	75 (46.6)	Handsewn	59 (56.2)
B3	74 (46.0)	Type of anastomosis	
Perianal lesions	69 (42.8)	Side-to-end	18 (18.0)
Anal ulceration, fissure	15 (9.3)	Side-to-side	54 (54.0)
Fistula/abscess	54 (33.5)	End-to-end	28 (28.0)
Medication at the time of surgery		Mean length of ileal resection (cm)	18.1 ± 17.1
5-ASA	24 (14.9)	Mean length of colonic resection (cm)	14.3 ± 17.7
Steroids	38 (23.6)	Mean length of digestive resection (cm)	31.6 ± 18.8
Budesonide	9 (5.6)	Perioperative complications	25 (16.8)
Thiopurines	36 (22.4)	Free margin resection	21 (17.1)
Methotrexate	5 (3.1)	Granuloma	47 (40.5)
Infliximab	15 (9.3)	Median CRP level, mg/L (IQR)	17.0 (3.8-61.0)

IQR: Interquartile; CRP: C-reactive protein; TNF: Tumor necrosis factor; IBD: Inflammatory bowel disease.

From the electronic database of the Pathology Department of the University Hospital Estaing of Clermont-Ferrand, France, we identified 205 patients who underwent an intestinal resection for CD, between 1986 and 2015, at the Institution. Only CD patients with a follow-up of at least 6 mo were considered for the study. Clinical, biological, pathological and endoscopic data were retrospectively collected from medical records (Table 1). As we aimed to be close to the real-life practice, we chose to include all the types of intestinal resection including patients with a definitive ostomy. For the patients with a temporary ostomy, the time point zero was defined as the time of the intestinal resection since we aimed to investigate all the potential factors influencing the time to recur (including type of resection and the presence of temporary or definitive ostomy). Surgical recurrence was defined as reoperation for CD. Clinical POR was defined, according to De Cruz *et al*[23], as recurrence of symptoms leading to hospitalization or therapeutic modifications after exclusion of other causes of recurrent symptoms such as bile-salt diarrhea, bacterial overgrowth and adhesion-related obstruction. Endoscopic POR was defined as Rutgeerts' score $\geqslant$ i2[12]. Regarding the endoscopies performed before the widespread of Rutgeerts' score use or with no score specified on the colonoscopy report, the score was evaluated retrospectively based on the content of the colonoscopy report. Patients underwent colonoscopies at their physician's discretion to assess potential subclinical disease. Patients were classified in endoscopy-based management (EBM) group if they underwent a systematic colonoscopy with no clinical POR at the time of endoscopy. All the patients included in the EBM group had a "step-up" therapeutic strategy in case of endoscopic POR[22]. The impact of the postoperative treatments was investigated in considering three groups: therapies to prevent endoscopic POR (treatment received during the period ranging from intestinal resection to endoscopic POR), therapies to prevent clinical POR (treatment received during the period ranging from endoscopic POR to clinical POR), and therapies to prevent surgical POR (treatment received during the period ranging from clinical POR to re-operation).

Data management and statistical analysis

Study data were collected and managed using REDCap electronic data capture tools hosted at Clermont-Ferrand University Hospital[27]. REDCap (Research



	1 yr	5 yr	10 yr	15 yr	20 yr
Surgical POR (%)	1.3	19.0	38.9	57.7	64.7
Clinical POR (%)	21.5	61.4	75.9	88.7	92.5
Endoscopic POR (%)	31.7	67.6	79.7	91.1	95.5

Figure 1 Kaplan Meir curves representing the prevalence of surgical, clinical and endoscopic postoperative recurrence in Crohn's disease patients undergoing intestinal resection in the Clermont-Ferrand inflammatory bowel disease unit (1986-2015).

Electronic Data Capture) is a secure, web-based application designed to support data capture for research studies, providing (1) an intuitive interface for validated data entry; (2) audit trails for tracking data manipulation and export procedures; (3) automated export procedures for seamless data downloads to common statistical packages; and (4) procedures for importing data from external sources.

Statistical analysis was performed using Stata 13 software (StataCorp LP, College Station, TX, United States). The tests were two-sided, with a type I error set at a = 0.05. Subject's characteristics were presented as mean ± SD or median (interquartile range) for continuous data (assumption of normality assessed using the Shapiro-Wilk test) and as the number of patients and associated percentages for categorical parameters. Comparisons between the independent groups were performed using the χ^2 or Fisher's exact tests for categorical variables, and using Student *t*-test or Mann-Whitney test for quantitative parameters (normality, assumption of homoscedasticity studied using Fisher-Snedecor test). Concerning the censored data, estimates were constructed using the Kaplan-Meier method. The log-rank test was used in a univariate analysis to test the prognostic value of patient characteristics for the occurrence of an event. Cox proportional hazards regression was used to investigate prognostic factors in a multivariate situation by backward and forward stepwise analysis of the factors considered significant in univariate analysis (entered into the model if $P < 0.10$) and according to clinically relevant parameters. The proportional hazard hypotheses were verified using Schoenfeld's test and plotting residuals. The interactions between possible predictive factors were also tested. Results were expressed as HRs and 95%CI.

RESULTS

Baseline characteristics of the patients

Overall, 161 CD patients were included in the study. The characteristics of these patients at the time of surgery are given in Table 1.

Prevalence of surgical, clinical and endoscopic POR

We observed a prevalence of endoscopic POR of 31.7%, 67.6%, 79.7%, 91.1% and 95.5%, respectively 1, 5, 10, 15 and 20 years after surgery (Figure 1). In our cohort, 21.5%, 61.4%, 75.9%, 88.7% and 92.5% of the patients experienced clinical POR at 1, 5, 10, 15 and 20 years, respectively (Figure 1). The rate of surgical POR was 1.3%, 19.0%, 38.9%, 57.7% and 64.7%, respectively 1, 5, 10, 15 and 20 years after surgery (Figure 1).

Risk factors of endoscopic POR

Among the 161 CD patients included in this study, 102 patients underwent a colonoscopy during their follow-up. The median interval for endoscopic POR was 2.0 years (0.6-3.6). While 54 patients (33.5%) received 5-ASA in prevention of endoscopic POR, 40 patients (24.8%), 7 patients (4.3%) and 41 patients (25.5%)

Table 2 Univariate analysis of risk factors for endoscopic postoperative recurrence in Crohn's disease

	Median time to endoscopic POR (mo)	HR [95%CI]	*P* value
Age		1.00 [0.99-1.00]	0.2
Age			
< 35 yr	41.4	Reference	
⩾ 35 yr	24.0	1.26 [0.86-1.84]	0.23
Age at diagnosis			
⩽ 16 yr	38.1	Reference	
16-40 yr	34.6	0.88 [0.47-1.67]	0.71
⩾ 40 yr	17.6	1.41 [0.60-2.63]	0.53
Tobacco use			
Non-smoker	38.1	Reference	
Active smoker	27.9	1.28 [0.77-1.70]	0.49
Previous intestinal resection			
No	43.5	Reference	
Yes	20.5	1.22 [0.98-2.15]	0.06
Total resection length > 50 cm			
No	20.5	Reference	
Yes	30.2	0.98 [0.56-1.73]	0.7
Disease behavior (Montreal classification)			
B1	-	Reference	
B2	43.5	1.30 [0.46-3.75]	0.62
B3	22.6	1.34 [0.47-3.80]	0.58
Fistulizing Crohn's disease (B3)			
No	29.3	Reference	
Yes	22.6	1.06 [0.73-1.53]	0.75
Perianal lesions			
No	34.6	Reference	
Yes	19.2	1.18 [0.82-1.71]	0.37
Type of perianal lesions			
Non-fistulizing lesions	33.4	Reference	
Fistula, abscess	20.5	1.10 [0.75-1.60]	0.23
Disease duration		1.00 [0.99-1.01]	0.77
Ileal resection > 50 cm			
No	25.9	Reference	
Yes	114.5	0.58 [0.21-1.60]	0.29
Prior exposure to anti-TNF therapy before surgery			
No	41.5	Reference	
Yes	8.0	3.91 [1.80-5.90]	< 0.001
Thiopurines therapy in prevention of endoscopic postoperative recurrence			
No	43.5	Reference	
Yes	43.7	1.07 [0.69-1.65]	0.75
Anti-TNF therapy in prevention of endoscopic postoperative recurrence			
No	41.4	Reference	
Yes	20.5	1.28 [0.78-2.13]	0.55
Period of surgery			
1986-1999		Reference	
2000-2015		1.00 [0.54-1.84]	0.99

CRP: C-reactive protein; TNF: Tumor necrosis factor.

were treated with thiopurines, methotrexate and anti-TNF, respectively. The postoperative endoscopic evaluation highlighted the following distribution: 19 patients (18.6%) classified as i0 according to the Rutgeerts' score[12], 19 patients (18.6%) as i1, 17 patients (16.7%) as i2, 12 patients (11.8%) as i3 and 35 patients (34.3%) as i4. In univariate analysis, prior intestinal resection, prior exposure to anti-TNF therapy before surgery seemed to be associated with shorter time until endoscopic POR (20.5 mo *vs* 43.5 mo, $P = 0.06$) and (8.0 mo *vs* 41.5 mo, $P < 0.001$), respectively (Table 2). Patients operated during the 1986-1999 period experienced earlier endoscopic POR than those operated during the 2000-2015 period ($P = 0.004$). In multivariate analysis, prior exposure to anti-TNF therapy before surgery (HR = 2.55, 95%CI: 1.37-4.73) and undergoing surgery during the 1986-1999 period (HR = 1.61, 95%CI: 1.04-2.49) were predictive of endoscopic POR.

Risk factors of clinical POR

Among the 161 included patients, the median time to clinical POR was 2.5 years (0.7-4.9). While 54 patients (33.5%) were treated with 5-ASA in prevention of clinical POR, 34 patients (21.1%), 2 patients (1.2%) and 26 patients (16.1%) were treated with thiopurines, methotrexate and anti-TNF, respectively. In univariate analysis, we reported that previous intestinal resection

Table 3 Univariate analysis of risk factors for clinical postoperative recurrence in Crohn's disease

	Median time to clinical POR (mo)	HR [95%CI]	*P* value
Age		1.00 [0.99-1.01]	0.4
Age			
< 35 yr	45.2	Reference	
⩾ 35 yr	30.2	1.25 [0.84-1.85]	0.27
Age at diagnosis			
⩽ 16 yr	38.1	Reference	
16-40 yr	48.0	0.81 [0.43-1.54]	0.52
⩾ 40 yr	30.2	1.03 [0.48-2.23]	0.93
Tobacco use			
Non-smoker	45.2	Reference	
Active smoker	43.7	1.00 [0.66-1.53]	0.98
Previous intestinal resection			
No	51.0	Reference	
Yes	26.6	1.62 [1.07-2.44]	0.02
Total resection length > 50 cm			
No	38.1	Reference	
Yes	33.4	1.20 [0.66-2.16]	0.55
Disease behavior (Montreal classification)			
B1	84.5	Reference	
B2	54.5	1.39 [0.48-4.00]	0.53
B3	28.9	1.61 [0.56-4.56]	0.37
Fistulizing Crohn's disease (B3)			
No	58.2	Reference	
Yes	28.9	1.21 [0.81-1.78]	0.34
Perianal lesions			
No	54.5	Reference	
Yes	26.9	1.26 [0.85-1.86]	0.24
Type of perianal lesions			
Non-fistulizing lesions	54.5	Reference	
Fistula, abscess	20.5	1.46 [1.01-2.16]	0.05
Disease duration		1.00 [0.99-1.01]	0.31
Ileal resection > 50 cm			
No	33.4	Reference	
Yes	59.5	0.72 [0.26-2.02]	0.54
Prior exposure to anti-TNF therapy before surgery			
No	48.0	Reference	
Yes	24.0	2.64 [1.24-4.33]	0.007
Thiopurines therapy in prevention of clinical postoperative recurrence			
No	44.8	Reference	
Yes	43.7	1.14 [0.74-1.76]	0.53
Anti-TNF therapy in prevention of clinical postoperative recurrence			
No	48.6	Reference	
Yes	29.3	1.39 [0.88-2.20]	0.16
Period of surgery			
1986-1999		Reference	
2000-2015		1.71 [1.12-2.63]	0.013

CRP: C-reactive protein; TNF: Tumor necrosis factor.

(51.0 mo *vs* 26.6 mo, *P* = 0.02), previous perianal fistula or abscess (54.5 mo *vs* 20.5 mo, *P* = 0.049) and prior exposure to anti-TNF therapy before surgery (24.0 *vs* 48.0, *P* = 0.007) were risk factors regarding clinical POR (Table 3). Patients operated during the 1986-1999 period experienced also earlier endoscopic POR than those operated during the 2000-2015 period (*P* = 0.013). In contrast, age at the time of surgery, age at the time of diagnosis, disease duration, tobacco use, resection length, CD behavior according to Montreal classification and all the other studied factors were not associated to an increased risk to experience clinical POR, in our cohort (Table 3). In addition, neither the use of thiopurines nor the use anti-TNF was protective factor of clinical POR. In multivariate analysis, previous intestinal resection (HR = 1.62, 95%CI: 1.07-2.46, *P* = 0.02), previous perianal abscess or fistula (HR = 1.50, 95%CI: 1.01-2.24, *P* = 0.042) and prior exposure to anti-TNF therapy before surgery (HR = 1.91, 95%CI: 1.01-3.66, *P* = 0.049) were predictive of clinical POR.

Risk factors of surgical POR

In our cohort (*n* = 161), the median time to surgical POR was 5.2 years (2.0-10.3). The medications used between the time of surgery and surgical POR were 5-ASA in 62 patients (38.5%), steroids in 78 patients (48.4%), thiopurines in 59 patients (36.6%) and anti-

Table 4 Univariate analysis of risk factors for surgical postoperative recurrence in Crohn's disease

	Median time to surgical POR (mo)	HR [95%CI]	*P* value
Age		1.00 [0.99-1.01]	0.29
Age			
< 35 yr	218.3	Reference	
⩾ 35 yr	131.6	1.32 [0.77-2.26]	0.30
Age at diagnosis			
⩽ 16 yr		Reference	
16-40 yr	144.0	1.37 [0.42-4.42]	0.60
⩾ 40 yr	108.6	1.73 [0.45-6.54]	0.42
Tobacco use			
Non-smoker	136.2	Reference	
Active smoker	173.4	0.84 [0.46-1.52]	0.56
Previous intestinal resection			
No	173.4	Reference	
Yes	108.6	1.74 [1.01-3.00]	0.04
Total resection length > 50 cm			
No	136.2	Reference	
Yes	120.0	1.50 [0.67-3.34]	0.32
Disease behavior (Montreal classification)			
B1		Reference	
B2	162.1	3.93 [0.52-29.33]	0.18
B3	128.8	5.71 [0.77-42.23]	0.09
Fistulizing Crohn's disease (B3)			
No	165.4	Reference	
Yes	128.8	1.78 [1.04-3.05]	0.03
Perianal lesions			
No	136.2	Reference	
Yes	151.1	0.99 [0.58-1.69]	0.97
Type of perianal lesions			
Non-fistulizing lesions	156.9	Reference	
Fistula, abscess	144.0	1.14 [0.66-1.97]	0.63
Disease duration		1.00 [0.99-1.01]	0.67
Ileal resection > 50 cm			
No	136.2	Reference	
Yes	120.0	1.23 [0.29-5.16]	0.78
Prior exposure to anti-TNF therapy before surgery			
No	151.1	Reference	
Yes	.	1.62 [0.92-7.08]	0.07
Thiopurines therapy in prevention of surgical postoperative recurrence			
No	144.0	Reference	
Yes	151.1	0.91 [0.50-1.65]	0.75
Anti-TNF therapy in prevention of surgical postoperative recurrence			
No	156.9	Reference	
Yes	136.2	2.09 [1.14-3.81]	0.02
Period of surgery			
1986-1999		Reference	
2000-2015		1.85 [1.22-2.80]	0.004

CRP: C-reactive protein; TNF: Tumor necrosis factor.

TNF in 69 patients (42.8%). In univariate analysis, previous intestinal resection (108.6 mo *vs* 173.4 mo, *P* = 0.04), fistulizing CD (B3 according to Montreal classification) (128.8 mo *vs* 162.1 mo, *P* = 0.03), prior exposure to anti-TNF therapy before surgery (*P* = 0.07) and anti-TNF therapy after surgery (136.2 mo *vs* 156.9 mo, *P* = 0.02) were associated with shorter time until reoperation (Table 4). The other potential risk factors investigated in the study were listed in Tables 1 and 2. In multivariate analysis, fistulizing CD (B3 according to Montreal classification) (HR = 1.78, 95%CI: 1.04-3.04, *P* = 0.003) and previous intestinal resection (HR = 1.7, 95%CI: 1.00-2.72, *P* = 0.05) were predictive of surgical POR.

Impact of an endoscopic-based management on the risk of POR

Overall, 49 of the 161 patients were included in the endoscopic-based management group. The median interval between initial surgery and endoscopy was 9.5 mo (6.0-22.9) in this group, including 63.2% of the patients (31/49) having a colonoscopy within the first year. Endoscopic POR occurred in 18 patients (36.7%) in the EBM-group. All of them underwent step-up therapeutic strategy as described in Table 5. In univariate analysis, an EBM was associated with a delayed time to clinical (33.4 mo *vs* 84.5 mo) and surgical recurrence. In multivariate analysis, an EBM decreased the risk of clinical POR (HR = 0.4, 95%CI:

Table 5 Step-up strategies in patients experiencing endoscopic postoperative recurrence in the endoscopic management-based group

Number of patient	Treatment before endoscopic evaluation	Rutgeerts' score	Treatment after endoscopic evaluation
1	None	i2	AZA
2	AZA	i3	IFX
3	AZA	i4	IFX
4	AZA	i2	AZA
5	AZA	i2	AZA (increased dose)
6	5-ASA	i4	IFX
7	ADA eow	i3	ADA ew
8	None	i4	ADA
9	5-ASA	i4	IFX + MTX
10	AZA	i4	IFX
11	AZA	i2	AZA (increased dose)
12	IFX + MTX	i3	IFX (increased dose) + MTX
13	ADA eow	i4	ADA ew
14	None	i2	AZA
15	None	i3	ADA
16	None	i2	AZA
17	None	i2	AZA
18	ADA eow	i3	ADA ew

AZA: Azathioprine; MTX: Methotrexate; IFX: Infliximab; ADA: Adalimumab; eow: Every other week; ew: Every week.

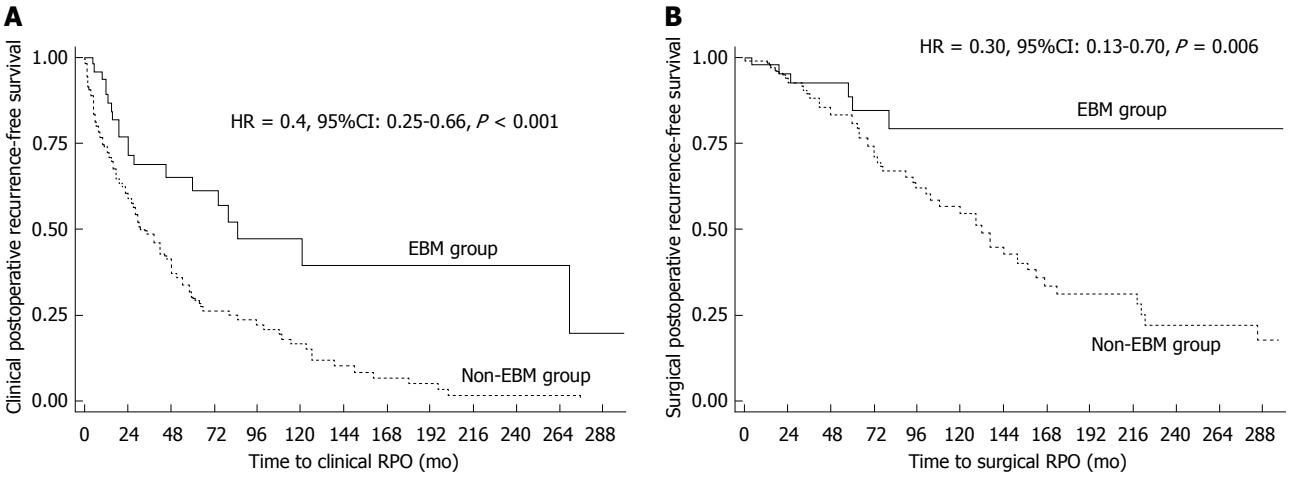


Figure 2 Long-term impact of endoscopic-based management on and clinical (A) and surgical (B) postoperative recurrence in Crohn's disease.

0.25-0.66, $P < 0.001$) (Figure 2A) and surgical POR (HR = 0.30, 95%CI: 0.13-0.70, $P = 0.006$) (Figure 2B).

DISCUSSION

Although performing a colonoscopy within the first year following surgery is commonly recommended in daily practice, the level of evidence suggesting that an EBM is an efficient strategy remains poorly investigated and is limited to short-term outcomes[23-26]. We reported here, the long-term impact of an EBM on the surgical and clinical POR risk that it has never been reported so far.

The prevalence of endoscopic POR in our cohort was perfectly in line with data from population-based cohort, which showed more than half of patients are experiencing endoscopic POR at 5 years, three quarters at 10 years and more than 90% at 15 years[3,28-30]. Our results also highlighted that more than three quarters (75.9%) of the patients experienced clinical POR within 10 years after surgery, that clinical symptoms occurred in almost all the CD patients followed in referral centers (92.5% at 20 years) and that almost two thirds (64.7%) of the CD patients were re-operated within 20 years of surgery. These data confirmed that surgery is not curative in CD in the large majority of the cases.

In our cohort, we confirmed that patients who underwent prior intestinal resection for CD, had higher risks of surgical, clinical and endoscopic POR, as previously showed in both population-based cohort[29] and referral centers[19]. In addition, we found that a fistulizing phenotype (B3 according to the Montreal classification) was associated with higher risk of endoscopic and surgical POR according to the results of a meta-analysis including 13 studies and 3044 patients (OR = 1.5, $P = 0.002$)[31] and several referral center-based studies[8]. Surprisingly, we did not show

any influence of tobacco use on the risk of POR in our cohort. However, smoking is often considered as the strongest risk factor for postoperative recurrence, increasing by twofold the risk of clinical recurrence and multiplying by 2.5 the risk for surgical POR within 10 years, with a dose-response relationship[21,32,33]. It could be partly explained by the retrospective design of our study and the fact that studying smoking habits is very difficult due to a wide modification of the smoking status during this long-term follow-up, the hardness to evaluate accurately the consumption of cigarettes and the underestimation of the number of smokers. Perianal disease is often admitted as predictor for POR. However, it remains unclear whether perianal lesions directly impacted the postoperative course of luminal disease or was only associated with perianal disease relapse leading to therapeutic modifications. In our cohort, the overall perianal lesions including both fistulizing and non-fistulizing (ulceration, fissure) lesions did not show any impact on the rate of recurrence. In contrast, we observed that prior perianal fistula or abscess was associated with increased clinical POR rate, but it did not influence the risk of both endoscopic and surgical POR. Most of the previous data indicated that perianal lesions were associated with clinical POR[28,34,35], while neither the studies investigating the risk factors for surgical POR[8,36,37], nor those interested in risk factors for endoscopic POR[8] achieved to prove the role of perianal involvement in the postoperative course of CD. Our results seemed to confirm that perianal involvement did not influence the risk of luminal recurrence, but underlined the fact that patients with perianal involvement had an increased risk of perianal symptomatic recurrence. Accordingly, we suggest that these patients require aggressive treatment after surgery, preferably to prevent perianal relapse rather than luminal recurrence, but this point warrants to be validated in additional studies. Some authors suggested using anti-TNF therapy in prevention of endoscopic POR in patients with prior exposure to anti-TNF before surgery[22]. This statement is based on experts' opinion rather than evidence-based medicine. However, in our cohort, we found that prior exposure to anti-TNF therapy before surgery is the most relevant risk factor for POR. It could mean that anti-TNF agents prescription associated to the most severe disease could predict an unfavorable postoperative course in CD. Stratifying the patients according to their risk factors of POR remains a key point in the management of the postoperative period in CD. However, the known risk factors do not allowed to accurately select the high-risk patients. Histologic factors, especially plexitis, could improve the selection of CD patients with ileocolic resection[38-41].

Although early colonoscopy after surgery is recommended in ECCO guidelines[21], low evidence supports this recommendation to date. Two retrospective studies evaluating the impact of postoperative EBM with tailored treatment according to the endoscopic findings did not report any benefit of this strategy on both clinical and surgical POR[23,24]. Bordeianou *et al*[24] reported no significant difference in time to clinical POR among the three following groups (n = 199 patients): immediate postoperative treatment, tailored treatment after endoscopy and no treatment. Similarly, De Cruz *et al*[23] reported no clinical benefit from an EBM in 136 CD patients. The authors explained their negative results in noting that the response to the endoscopic findings was not standardized and immunosuppressive therapy was uncommon during their study period. More recently, among 132 operated on for CD from the Saint-Louis Hospital, Paris, France, the authors reported a decreased clinical POR rate 5 years after surgery, in the patients with EBM, compared to the non-EBM group (26% *vs* 52%)[25]. Recently, the landmark POCER trial, a prospective, well-designed study, compared the impact of a tailored management according to clinical risk of recurrence, with early colonoscopy and treatment step-up on recurrence[26]. The results showed that an early EBM, performed 6 mo after surgery, decreased the rate of endoscopic POR at 18 mo[26]. However the long-term impact of EBM on the risk of POR (especially surgical) remained unknown. Our results indicated for the first time that an EBM influenced the risk of reoperation for CD, leading to a delayed time before surgical POR. In addition, we confirmed that the EBM group experienced less clinical POR over time than the non-EBM group, in a long period of follow-up. As our cohort overlapped a very long period with different available medications overtime, we did not show any impact of the postoperative treatment, especially biologics, in this population.

The main limits of this study were the retrospective and monocentric design. In addition, the time of endoscopy (median = 9.5 mo after surgery) and the step-up strategy were not standardized for all the patients. However, we observed for the first time the positive impact of an EBM on the risk of reoperation in CD, in a cohort monitored during almost 30 years (1986-2015) and based on a Pathology Department electronic database (consecutive patients).

In conclusion, POR is very frequent in CD and remains a critical issue in the management of the postoperative period. The identification of predictors to select the high-risk patients warranting top-down strategy in the postoperative period is crucial. An endoscopic-based management within the first year after surgery decreases the risk of symptoms recurrence and reoperation and then have to be recommended in daily practice.

ACKNOWLEDGMENTS

Thank you to the company "MG translate" for reviewing the manuscript.

COMMENTS

Background

Surgical resection is unfortunately not curative in Crohn's disease (CD), and postoperative recurrence (POR) remains a crucial issue in these patients. Performing an endoscopy within the first year after surgery is often recommended in clinical practice. However, the level of evidence suggesting the efficacy of such strategy remains low. Two retrospective studies reported no impact of an endoscopy-based management (EBM). A French group suggested, in a retrospective cohort, that an EBM was associated with a decrease risk of clinical POR at 5 years. Recently, the landmark POCER trial showed that an early EBM decreased the risk of endoscopic POR at 18 months post-surgery. However the long-term impact of EBM on the risks of clinical and surgical POR remains unknown.

Research frontiers

The level of evidence suggesting the efficacy of an endoscopy-based strategy in CD remains low especially in the long-term. In the present study, the authors aimed to investigate whether an endoscopy-based strategy could prevent the long-term risk of POR in CD.

Innovations and breakthroughs

This paper showed for the first time, that an endoscopic-based management within the first year after surgery decreases the long-term risk of symptoms recurrence and reoperation.

Applications

Endoscopy-based management should be recommended in all CD patients within the first year after surgery in daily practice as it highly decreases the long-term risk of clinical recurrence and reoperation.

Terminology

An endoscopy-based strategy in CD means treatment intensification in case of endoscopic recurrence to prevent symptoms reappearance.

Peer-review

This article deals with an important aspect of CD- post operative recurrence. The article is well written in general.

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DOI: 10.3748/wjg.v22.i21.5079

World J Gastroenterol 2016 June 7; 22(21): 5079-5087
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

ORIGINAL ARTICLE

Retrospective Cohort Study

Ulcerative colitis patients in clinical remission demonstrate correlations between fecal immunochemical test results, mucosal healing, and risk of relapse

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Author contributions: All authors contributed to this paper.

Institutional review board statement: This study was reviewed and approved for publication by our Institutional Reviewer.

Informed consent statement: All study participants or their legal guardians provided informed written consent regarding personal and medical data collection prior to study enrollment.

Conflict-of-interest statement: All authors have no conflicts of interest related to the manuscript.

Data sharing statement: The original anonymous dataset is available upon request from the corresponding author at sakikoh@cc.okayama-u.ac.jp.

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Received: March 13, 2016
Peer-review started: March 13, 2016
First decision: March 21, 2016
Revised: March 31, 2016
Accepted: May 4, 2016
Article in press: May 4, 2016
Published online: June 7, 2016

Abstract

AIM: To assess the risk of relapse in ulcerative colitis (UC) patients in clinical remission using mucosal status and fecal immunochemical test (FIT) results.

METHODS: The clinical outcomes of 194 UC patients in clinical remission who underwent colonoscopy were based on evaluations of Mayo endoscopic subscores (MESs) and FIT results.

RESULTS: Patients with an MES of 0 (n = 94, 48%) showed a ten-fold lower risk of relapse than those with an MES of 1-3 (n = 100, 52%) (HR = 0.10, 95%CI: 0.05-0.19). A negative FIT result (fecal hemoglobin concentrations $\leqslant$ 100 ng/mL) was predictive of patients with an MES of 0, with a sensitivity of 0.94 and a specific of 0.76. Moreover, patients with a negative FIT score had a six-fold lower risk of clinical relapse than those with a positive score (HR = 0.17, 95%CI: 0.10-0.28). Inclusion of the distinguishing parameter, sustaining clinical remission > 12 mo, resulted in an even stronger correlation between negative FIT results

and an MES of 0 with respect to the risk of clinical relapse (HR = 0.11, 95%CI: 0.04-0.23).

CONCLUSION: Negative FIT results one year or more after remission induction correlate with complete mucosal healing (MES 0) and better prognosis. Performing FIT one year after remission induction may be useful for evaluating relapse risk.

Key words: Ulcerative colitis; Clinical remission; Mucosal healing; Mayo endoscopic subscore; Quantitative fecal immunochemical test

Core tip: Mucosal healing has been recognized as the treatment goal of. In this study, the relapse rate differed greatly between patients with a Mayo endoscopic subscore (MES) of 0 and an MES of 1 such that mucosal healing should be defined as an MES of 0. We previously reported that a negative fecal immunochemical test (FIT) correlates positively with mucosal healing. This paper indicated that patients with a negative FIT demonstrated a lower risk of clinical relapse than those with a positive FIT and that the risk of relapse in patients in prolonged remission and with a negative FIT was equivalent to that of patients with an MES of 0.

Nakarai A, Kato J, Hiraoka S, Takashima S, Takei D, Inokuchi T, Sugihara Y, Takahara M, Harada K, Okada H. Ulcerative colitis patients in clinical remission demonstrate correlations between fecal immunochemical test results, mucosal healing, and risk of relapse. *World J Gastroenterol* 2016; 22(21): 5079-5087 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i21/5079.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i21.5079

INTRODUCTION

Ulcerative colitis (UC) is an idiopathic chronic inflammatory disorder that, when untreated, results in symptoms of diarrhea and bloody stool. Current studies evaluating UC treatment using colonoscopy cite a need to achieve not only clinical responses but also mucosal healing, which is associated with sustained clinical remission and reduced rates of hospitalization and surgical resection[1]. An additional study indicated that early mucosal healing after the administration of infliximab for UC correlates with improved clinical outcomes, including the avoidance of colectomy[2]. Another report showed that a lack of mucosal healing after initial corticosteroid therapy is associated with late negative outcomes[3].

Nevertheless, standardized criteria for evaluating disease severity and the degree of mucosal healing are not presently available[4]. Some reports define mucosal healing as an MES of 0 or 1[2,5], whereas other reports consider only an MES of 0 to be healing[6]. Such inconsistencies complicate interpretations of the significance of mucosal healing in the treatment of UC such that differences in long-term prognosis (evaluated by relapse of clinical symptoms and/or colectomy) between clinically asymptomatic patients with an MES of 0 (complete mucosal healing) and those with an MES of 1 (partial mucosal healing) are rarely reported.

Using colonoscopy to evaluate mucosal status in UC patients is expensive and invasive. Previous work reported by our group demonstrates that a quantitative fecal occult blood test (FIT) effectively reflects the mucosal status of patients with UC and that a negative FIT correlates strongly with mucosal healing[7]. Although we found a significantly higher positive correlation between negative FIT results and an MES of 0 (> 90%) compared with an MES of 0 or 1 (< 60%), the likelihood of relapse in patients in remission with a negative FIT has not been formally evaluated.

In this study, we retrospectively reevaluated and subdivided the colonoscopic findings of UC patients in clinical remission into subcategories of MES 0 or MES 1. Patient prognoses (relapse of clinical symptoms and colectomy rate) were evaluated to determine whether the optimal goal of UC treatment should be either an MES of 0 or 1 or only an MES of 0. Correlations between FIT results and MES in these patients were also evaluated to determine whether FIT scores can function as a surrogate marker for meeting the treatment goals of UC patients in clinical remission.

MATERIALS AND METHODS

Patients

Between January 2006 and January 2014, ambulatory UC patients who were making periodic visits to Okayama University Hospital were requested to prepare and bring fecal samples to scheduled colonoscopy appointments for an evaluation of disease activity and surveillance. Fecal samples (prior to colonoscopy bowel preparation) were tested for fecal occult blood with an FIT, and the results were evaluated with regard to colonoscopic findings. All of the patients had an established diagnosis of UC according to endoscopic and histologic assessments and had received medical therapy.

Clinical disease activity was scored using the Mayo score, which is based on the following four criteria: stool frequency, rectal bleeding, endoscopic findings, and physician global assessment (0, normal; 1, mild disease; 2, moderate disease; 3, severe disease)[8]. Clinical remission was defined as a partial Mayo score (Mayo score without endoscopic findings) $\leqslant$ 2 points, with no individual subscore exceeding 1 point[5]. Clinical relapse was defined by an increase or modification of concomitant medications due to a worsening of symptoms. Patients in clinical remission at the time of colonoscopy were considered eligible

for this retrospective cohort study. The study protocol was approved by the Institutional Review Board of Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences.

Fecal sampling and instrument for FIT analysis

The details of the method used for the FIT were described previously[9-11]. Briefly, patients prepared fecal samples before bowel preparation for colonoscopy using an OC-Hemodia sampling probe (Eiken Chemical, Tokyo, Japan) provided by the manufacturer of the kit. An 8 cm × 2 cm test tube-shaped container holds the sampling probe. The patient inserts the probe into several different areas of stool and then firmly places it back into the tube for sealing. The probe tip with the fecal sample is suspended in a standard volume of hemoglobin-stabilizing buffer. Submitted stool samples were immediately processed and examined using OC-SENSOR neo (Eiken Chemical), which can accurately measure fecal hemoglobin concentrations of 50 ng/mL to 1000 ng/mL. Fecal specimens with a hemoglobin concentration over 1000 ng/mL were measured following dilution. Because FIT results are inaccurate at hemoglobin concentrations below 50 ng/mL, specimens with a hemoglobin concentration in this range were categorized as one (0-50 ng/mL).

Colonoscopy

On the day of the colonoscopy, patients received a polyethylene glycol-based or magnesium citrate-based electrolyte solution for bowel preparation and ingested it according to the manufacturer's instructions. After colonic lavage, the patients underwent colonoscopy. Patients were excluded if the colonoscopic examination was incomplete due to problems with the bowel preparation or if the colonoscope could not be inserted into the cecum. At colonoscopy, the colonoscopists were not blinded to the clinical data. However, at data collection for analysis, colonoscopic images were re-evaluated by experienced colonoscopists who did not know the clinical data.

The mucosal status of UC was assessed using the MES classification system. Evaluation was performed at each portion of the colorectum (cecum; ascending, transverse, descending and sigmoid colon; and rectum), and the maximum score in the colorectum of each patient was used for analysis. An MES of "0" throughout the colorectum was defined as complete mucosal healing, whereas a maximum MES of "1" in the colorectum was defined as partial mucosal healing.

Statistical analysis

Statistical analyses were performed using JMP version 9 (SAS Institute, Cary, NC, United States). A Kaplan-Meier curve estimating the duration of sustained clinical remission was generated for each predefined patient group, and comparisons between groups were performed using a 2-sided log-rank test. The Cox proportional hazards regression model was used to calculate hazard ratios (HRs) between groups, quantifying the likelihood of clinical relapse using a 95%CI. Comparative analyses, such as a χ^2 test and the Mann-Whitney test, were used for cross-sectional analysis of categorical data. Spearman rank correlation was performed to measure the association between fecal hemoglobin concentrations and MES, and trends between these values were evaluated using the Cochran-Armitage trend test. A receiver operating characteristic (ROC) curve was generated to estimate appropriate cutoff values for the FIT. The area under the curve (AUC), and sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV), for detecting mucosal healing based on the FIT results was calculated. All *P*-values were two-sided and considered significant when less than 0.05.

RESULTS

Clinical characteristics of the patients

A total of 248 UC patients who underwent colonoscopy between January 2006 and January 2014 also underwent a corresponding FIT. If a patient underwent two or more colonoscopies during remission, then only the data from the first colonoscopy were included. Among these patients, 194 (78%) demonstrated clinical remission at the time of colonoscopy and were enrolled in the study.

Table 1 summarizes the clinical characteristics of the 194 patients (99 male and 95 female; median age at UC onset 32 years). At the time of colonoscopy, the median duration of sustaining clinical remission was 11 mo. Colonoscopic findings revealed that the maximum MES was 0 in 94 (49%) cases, 1 in 57 (29%) cases, 2 in 39 (20%) cases, and 3 in 7 (2%) cases. On the basis of our definitions, 94 and 57 patients had complete and partial mucosal healing, respectively. Among the 194 patients, 111 (57%) cases showed fecal hemoglobin concentrations of 100 ng/mL or lower and were defined as FIT-negative.

Difference in the prognosis of UC patients according to the MES

Kaplan-Meier curves comparing the maintenance of clinical remission among patients with an MES of 0-3 are shown in Figure 1. There was a statistically significant difference in remission maintenance rates between each MES group ($P < 0.0001$, log-rank test). The Cox proportional hazards model suggested that patients with an MES of 1 were more than seven times more likely to relapse than patients with an MES of 0 (HR of MES 1 *vs* MES 0, 7.40; 95%CI: 3.78-15.06). Conversely, MES 0 patients were approximately ten times less likely to relapse than MES 1-3 patients (HR = 0.10; 95%CI: 0.05-0.19). Furthermore, the risk of colectomy or the occurrence of dysplasia/cancer did not vary significantly between patients in different

Table 1 Incidence of clinical characteristics among participants

Total	194
Gender, *n* (%)	
Male	99 (51)
Female	95 (49)
Extent of disease, *n* (%)	
Pancolitis	125 (64)
Left-side colitis	48 (25)
Proctitis	21 (11)
Median (IQR) age at onset	32 (22-43)
Median (IQR) duration of disease, months	107 (51-194)
Median (IQR) age of undergoing colonoscopy	44 (33-56)
Median (IQR) duration of sustaining clinical remission at the time of colonoscopy, months	11 (6-23)
Concomitant medications, *n* (%)	
Aminosalicylate	178 (92)
Corticosteroids	27 (14)
Mercaptopurine/Azathioprine	79 (41)
Tacrolimus	10 (5)
Biologics	9 (5)
Colonoscopy findings, *n* (%) (maximum index in the colorectum)	
MES 0	94 (49)
MES 1	57 (29)
MES 2	39 (20)
MES 3	4 (2)
Fecal Hb concentrations (ng/mL), *n* (%)	
0-100	111 (57)
101-1000	56 (29)
1001-10000	20 (10)
10001-	7 (4)

MES: Mayo Endoscopic subscore; Hb: Hemoglobin; IQR: Interquartile range.

MES groups (data not shown). Overall, these results suggest that the treatment goal for minimizing relapse in UC patients in clinical remission should be to achieve a score of MES 0, rather than MES 1.

Comparison of clinical characteristics in MES 0 patients relative to MES 1-3 patients

We have shown that achievement of complete mucosal healing (MES 0) is optimal for UC patients with regard to the maintenance of clinical remission. We also compared other clinical characteristics of the 94 patients in clinical remission with complete mucosal healing (MES 0) with those of 100 patients who showed only partial healing (MES 1) or more inflammation (MES 2, 3) (Table 2). The former subgroup had maintained clinical remission for a significantly longer time at the time of colonoscopy (17 mo *vs* 9 mo, $P < 0.0001$) and was administered mercaptopurine/azathioprine more frequently than the latter (45 patients *vs* 34 patients, $P = 0.049$). The FIT results demonstrated that fecal hemoglobin concentrations were significantly lower (50 ng/mL *vs* 315 ng/mL, $P < 0.0001$) in patients with complete mucosal healing than in patients with partial healing or more inflammation.

Applicability of FIT results for predicting complete mucosal healing in UC patients in clinical remission

Our data demonstrated that MES 0 patients in clinical remission presented significantly lower fecal hemoglobin concentrations than MES 1-3 patients. The correlation between FIT results and colonoscopic findings among these patient subgroups is illustrated in Figure 2. The Spearman rank correlation coefficient quantifying the relationship between FIT values and MES subgroups was 0.6530 ($P < 0.0001$). The proportion of cases with fecal hemoglobin concentrations ⩽ 100 ng/mL was greatest in the MES 0 patients (88/94, 92%), and decreased gradually as the MES increased (MES 1: 16/57, 28%; MES 2: 6/39, 15%). The trend of the decrease in the relationship

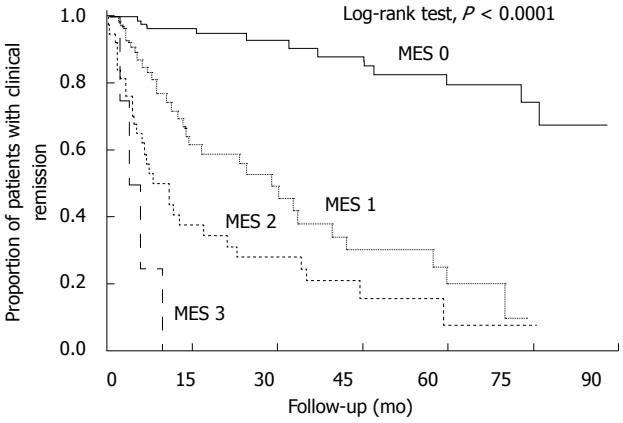

Figure 1 Kaplan-Meier curves depicting the rates of clinical remission maintenance with regard to the Mayo endoscopic subscores. There were statistically significant differences in the cumulative remission maintenance rates between patients in each Mayo endoscopic subscore (MES) subgroup ($P < 0.0001$, log-rank test). The hazard ratio for risk of relapse of patients with an MES of 0 relative to those with an MESs of 1-3 was 0.10 (95%CI: 0.05-0.19).

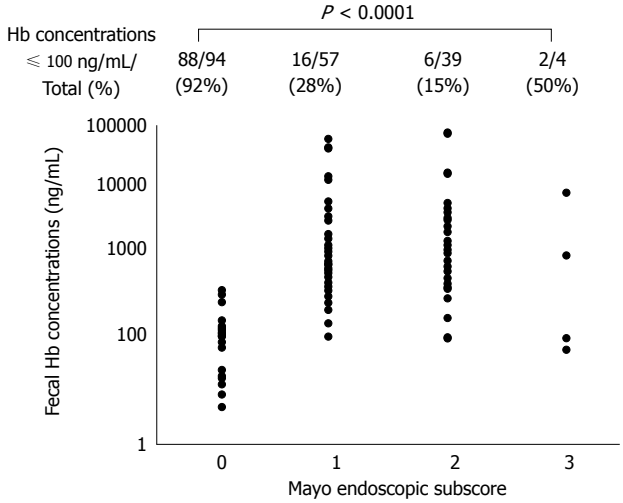

Figure 2 Correlation between fecal immunochemical test results and the Mayo endoscopic subscores. There was a significant positive correlation between fecal hemoglobin concentrations and the MES (Spearman rank correlation coefficient = 0.6530, $P < 0.0001$). The proportion of cases with negative FIT results (fecal hemoglobin concentration ⩽ 100 ng/mL) was greatest in cases with an MES of 0 (88/94, 92%). The proportion decreased gradually as the MES increased (MES 1: 16/57, 28%, MES 2: 6/39, 15%), and the trend of the decrease in relation to the MES was statistically significant ($P < 0.0001$, Cochran-Armitage trend test).

Table 2 Characteristics of patients with MES 0 *vs* MES 1-3

Characteristics	MES 0 (*n* = 94)	MES 1-3 (*n* = 100)	*P* value
Gender, *n* (%)			
Male	54 (57)	45 (45)	0.083
Female	40 (43)	55 (55)	
Median age, yr (IQR)	46 (32-60)	41 (33-51)	0.051
Median duration of disease, mo (IQR)	99 (53-198)	112 (44-191)	0.950
Median age at onset, yr (IQR)	34 (22-47)	31 (22-40)	0.130
Median duration of sustaining clinical remission at the time of colonoscopy, mo (IQR)	17 (9-33)	9 (4-13)	< 0.0001
Extent of disease, *n* (%)			
Pancolitis	59 (63)	66 (66)	0.340
Left-side colitis	27 (29)	21 (21)	
Proctitis	8 (8)	13 (13)	
Concomitant medications, *n* (%)			
Aminosalicylate	86 (91)	92 (92)	0.900
Corticosteroids	11 (12)	16 (16)	0.390
Mercaptopurine/ Azathioprine	45 (48)	34 (34)	0.049
Tacrolimus	4 (4)	6 (6)	0.580
Biologics	2 (2)	7 (7)	0.110
Fecal Hb concentrations (ng/mL)	50 (4-50)	315 (108-1277)	< 0.0001

MES: Mayo endoscopic subscore; Hb: Hemoglobin; IQR: Interquartile range.

to the MES was statistically significant ($P < 0.0001$, Cochran-Armitage trend test).

Figure 3 shows the ROC curve for fecal hemoglobin concentrations in relation to complete mucosal healing. A cutoff value of 100 ng/mL was shown to effectively differentiate between patients with and without complete mucosal healing at a sensitivity of 0.94 and a specificity of 0.76. The PPV of applying 100 ng/mL as a cut-off for determining complete mucosal healing was 0.79, whereas the NPV was 0.93. The corresponding AUC was 0.88.

In addition to the results relating FIT to mucosal healing, Kaplan-Meier curves showed that the cumulative remission maintenance rate was also significantly different between patients with fecal hemoglobin concentrations ⩽ 100 ng/mL and those with fecal hemoglobin concentrations > 100 ng/mL ($P < 0.0001$, log-rank test; Figure 4). The Cox proportional hazards model indicated that patients with a negative FIT value had a six-fold lower risk of clinical relapse than those with a positive FIT (HR = 0.17; 95%CI: 0.10-0.28).

Differences between MES 0 and MES 1-3 FIT negative patients

In UC patients in clinical remission, negative FIT values correlate closely with an MES of 0, and patients in either clinical category demonstrate a better prognosis. However, as a marker for a reduced risk of UC relapse, a rating of MES 0 is slightly more accurate in predicting risk than a negative FIT value (HR = 0.10 *vs* 0.17). In a comparison between Kaplan-Meier curves of MES

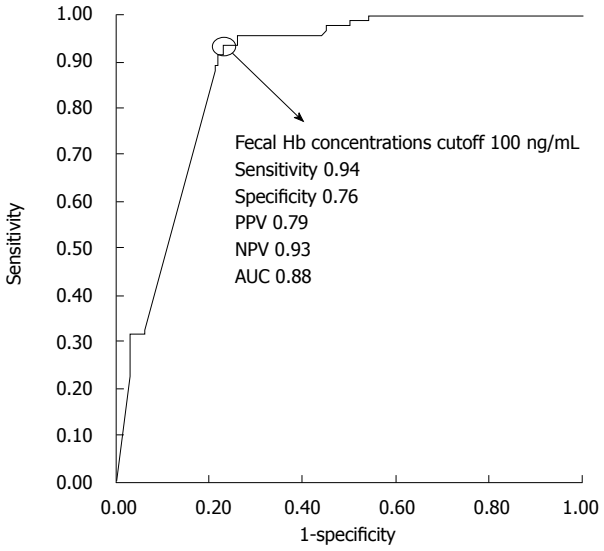

Figure 3 Receiver operating characteristic curve of fecal hemoglobin concentrations for predicting complete mucosal healing. A cutoff value of 100 ng/mL differentiated between patients with or without complete mucosal healing with the following values: 0.94 sensitivity, 0.76 specificity, 0.79 PPV, 0.93 NPV, and 0.88 accuracy. The corresponding area under the curve was 0.88.

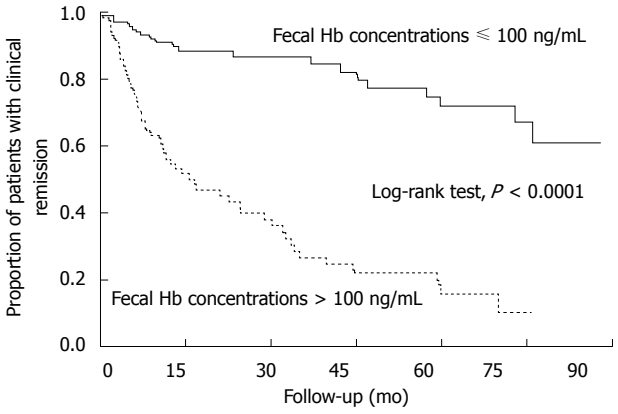

Figure 4 Kaplan-Meier curves depicting maintenance of clinical remission with regard to fecal immunochemical test results. There was a statistically significant difference in cumulative remission maintenance rates between the patients with a negative FIT result and those with a positive FIT result ($P < 0.0001$, log-rank test). The hazard ratio relating the relapse risk in patients with a negative FIT to those with a positive FIT was 0.17 (95%CI: 0.10-0.28).

0 (Figure 1) and negative FIT (Figure 4), the relapse rate within one year after colonoscopy/FIT was slightly higher in patients with a negative FIT than in those with an MES of 0 (1 year relapse rate 9% *vs* 3%, and 5 year relapse rate 22% *vs* 17%, respectively). In addition, the duration of sustaining clinical remission at colonoscopy/FIT was significantly longer in patients with an MES of 0 than in those with an MES of 1-3 among patients with a negative FIT (16 mo *vs* 8 mo, $P = 0.001$). These findings suggest that patients who enter clinical remission are more likely to demonstrate a negative FIT first (cessation of colorectal bleeding), followed by complete mucosal healing.

Because the MES 0 patients sustained clinical

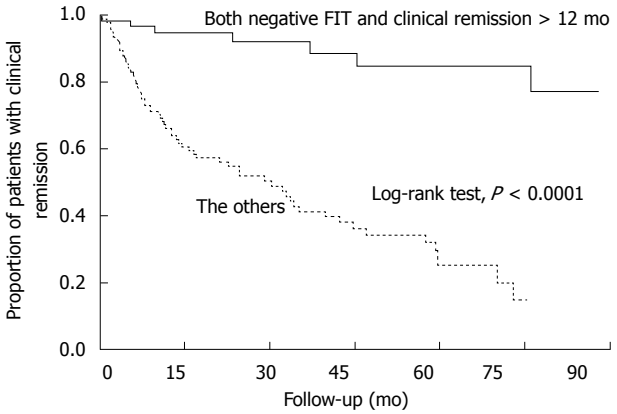

Figure 5 Kaplan-Meier curves depicting the maintenance of clinical remission in patients with both a negative fecal immunochemical test and clinical remission > 12 mo relative to all other patients. There was a statistically significant difference in the cumulative remission maintenance rates between patients with both a negative FIT and clinical remission > 12 mo (n = 66) and all other patients (n = 128) (P < 0.0001, log-rank test). The hazard ratio relating relapse risk in patients with both a negative FIT and clinical remission > 12 mo to relapse risk in all other patients was 0.11 (95%CI: 0.04-0.23).

remission for a longer time than the MES 1-3 patients, we performed multivariate analysis and found that clinical remission > 12 mo was a significant factor for predicting an MES of 0 among our 194 subjects (OR = 8.47; 95%CI: 3.33-24.03). Thus, we used Kaplan-Meier curves to illustrate the relationship between the patients who fulfilled both classifiers - negative FIT and clinical remission > 12 mo - and all others (Figure 5). The Cox proportional hazards model indicated that patients with both a negative FIT and clinical remission > 12 mo have a nine-fold lower risk of relapse than all other patients (HR = 0.11, 95%CI: 0.04-0.23). The one-year and five-year relapse rates for this group (FIT negative, remission > 12 mo) were similar to those of patients with MES 0 (4% and 15%, respectively). Taken together, these findings suggest that a negative FIT in patients who have sustained clinical remission for at least one year is a good indicator of complete mucosal healing and a predictor of low relapse risk.

Figure 6 indicated the proposed workflow of the follow-up of UC patients using FIT. During remission induction therapy, we recommend to measure FIT about once 2-4 wk, comparing those results to the baseline FIT result. When FIT results decrease, we make therapy maintained or weakened. On the other hand, when FIT results do not decrease or increase, therapy should be considered to intensify. After remission induction, we recommend to measure FIT every visit. Since patients with both negative FIT and clinical remission > 12 mo are highly probable to have achieved mucosal healing with low risk of relapse, these patients could be followed with longer intervals. Otherwise, patients are considered to have residual inflammation with considerable risk of relapse, they need to be followed up closely.

DISCUSSION

In this study, our goal was to distinguish which MES scores represent optimal mucosal healing - an MES of 0 alone or an MES of 0 or 1. In addition, because we previously reported that FIT can function as a predictor of mucosal status in UC patients, we further discriminated the predictability of the FIT as a measure of mucosal status and of the prognosis of patients with clinical remission. In retrospective analyses of the differences in long-term clinical outcomes between patients with an MES of 0 and those with an MES 1, we found that negative FIT results were significantly more likely in patients with complete mucosal healing (MES 0) than in patients with partial mucosal healing (MES 1). Our findings showed that patients with an MES of 0 alone were much less likely to relapse and that negative FIT results showed a stronger positive correlation with an MES of 0 alone than with an MES of 0 or 1, as well as with a reduced risk of relapse. Moreover, analyses including the parameter "sustaining clinical remission > 12 mo" revealed a more robust correlation between negative FIT results and complete mucosal healing with regard to the minimum risk of relapse.

Standardized criteria for evaluating the severity of ulcerative colitis and for defining mucosal healing in patients with UC have yet to be established[4]. Many prior clinical studies have defined mucosal healing as maintaining an MES of 0 or 1[2,5], and there are few long-term studies distinguishing the ability of an MES of 0 or an MES of 1 to contribute to the maintenance of clinical remission in UC patients. Detailed analysis of findings from the Active Ulcerative Colitis Trials (ACT-1, 2)[2] showed that the clinical remission rate at 54 wk from the time of colonoscopy (performed after induction of remission at week 8) was 73% in MES 0 patients and 47% in MES 1 patients; more than half of the MES 1 patients relapsed. In addition, other groups including our group, reported that patients in clinical remission with an MES of 0 were less likely to relapse than those with an MES of 1 or more, using a retrospective cohort[12,13]. In contrast, in a study evaluating the effectiveness of mesalazine for maintaining UC remission, Meucci *et al*[6] reported no significant difference between the rates of relapse at one year in MES 0 *vs* MES 1 patients.

Negative FIT results among the subjects in clinical remission also correlated closely with an MES of 0, and the patients in clinical remission who showed a negative FIT result were less likely to relapse than those with a positive FIT. These results suggest that a negative FIT result may function as a surrogate marker for complete mucosal healing and should be the treatment goal for UC patients in remission.

Nevertheless, our data do not show a complete overlap between the clinical behavior of patients with

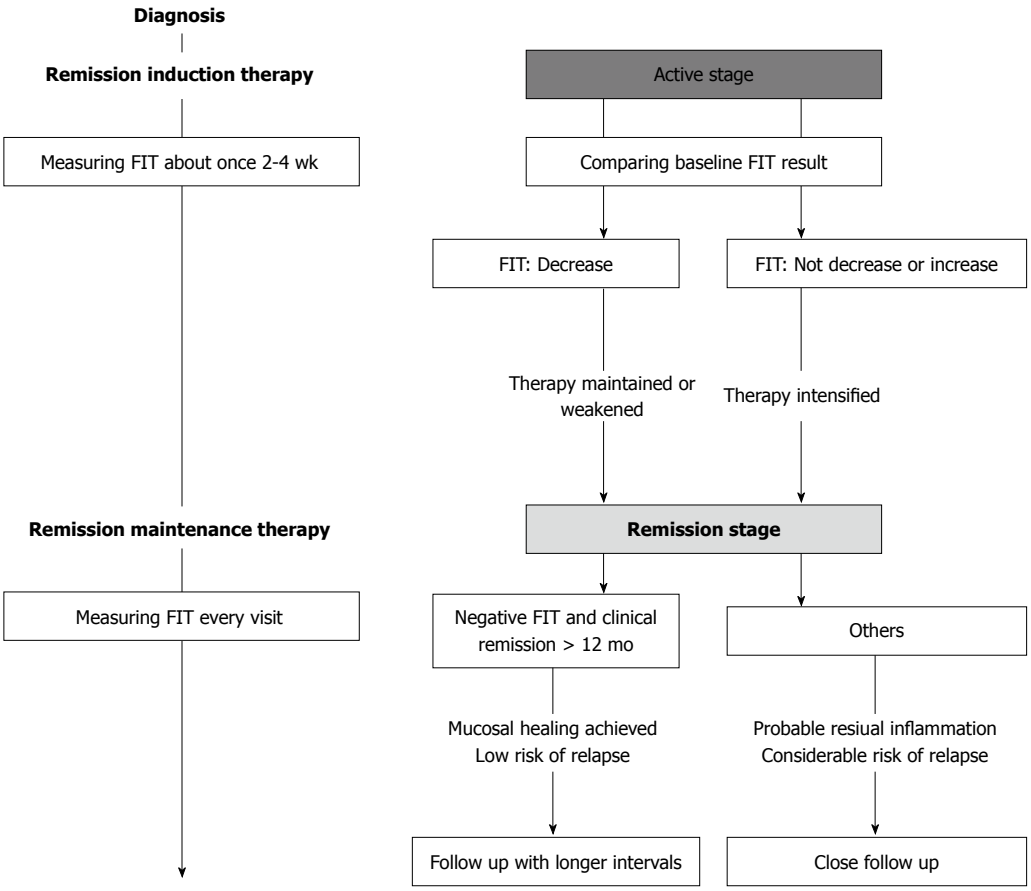

Figure 6 Proposed workflow of the follow-up of Ulcerative colitis patients using fecal immunochemical test. FIT: Fecal immunochemical test.

negative FIT results and that of MES 0 patients as the former are more likely to relapse within one year after colonoscopy/FIT than the latter. In addition, MES 0 patients with a negative FIT at colonoscopy/FIT maintained clinical remission significantly longer than MES 1-3 patients. These results suggest that UC patients who enter clinical remission achieve a negative FIT first, followed by an MES of 0 (complete healing). The relapse rate among patients with a negative FIT who sustained clinical remission for one year or more was the same as the rate among MES 0 patients.

On the basis of our findings, we recommend that UC patients undergo an FIT one year after induction of clinical remission. If the FIT result is negative, then colonoscopy can be safely skipped, and the optimal treatment goal of complete mucosal healing should be considered met. If the FIT result is positive, then physicians should consider performing a colonoscopy or intensifying treatment. Thus, the FIT (an easy, non-invasive and low-cost test) may function advantageously as a substitute for endoscopy to measure mucosal status and risk of relapse in UC patients one year after remission.

There is accumulating evidence that fecal calprotectin, a major protein found in the cytosol of inflammatory cells, is an effective pioneer and is useful for assessing intestinal inflammation[14-16]. Several studies have reported that fecal calprotectin values can predict relapse in UC patients in clinical remission[17-21]. Although these reports indicated that patients with higher fecal calprotectin levels were more likely to relapse within several to 12 mo, no correlation between fecal calprotectin and mucosal status was identified because endoscopic examinations were not included in the studies.

Other reports, which indicate that fecal calprotectin levels can predict endoscopic mucosal healing[22-24], did not investigate risk of relapse. Thus, clear evidence defining the relationship between fecal markers, mucosal status and risk of relapse is lacking. In contrast, our analyses of FIT results as a marker for complete mucosal healing include all 3 variables: Negative FIT results one year or more after UC remission correlated with complete mucosal healing and also with a minimum risk of relapse. Because fecal calprotectin has recently been reported to also correlate with the presence of histological inflammation[25], testing the correlation between negative FIT results and histological remission is one of our future aims.

Reports comparing fecal hemoglobin and calprotectin levels directly as predictors of mucosal status are scarce. Mooiweer *et al*[26] demonstrated that both markers are similarly effective in identifying inflammatory bowel disease (IBD) patients with active endoscopic inflammation. However, to the best

of our knowledge, no reports have compared the predictability of mucosal healing and/or risk of relapse between the two fecal markers. To further understand the roles of these markers in the clinical management of IBD, we aim to conduct such comparative studies in the future.

The FIT has particular advantages over fecal calprotectin testing: Fetal calprotectin is measured using an enzyme-linked immunosorbent assay (ELISA), which is time-consuming and requires specialized techniques, whereas the FIT can be easily measured automatically in a few minutes. In addition, there is significant inter- and intra-assay variability in measures of fecal calprotectin levels using different ELISA diagnostic kits [such as PhiCal Calprotectin ELISA (R-Biopharm, Darmstadt, Germany), Calprest (Eurospital, Trieste, Italy), and Calprotectin ELISA (Bühlmann, Basel, Switzerland)]. The lack of an established assay kit and an optimal cutoff value for detecting mucosal healing/inflammation in UC patients is another major limitation of fecal calprotectin[14-16]. In this regard, the FIT used in this study is the most widely used system worldwide (OC-Sensor neo), and it maintains a consistent standard cutoff (100 ng/mL) for CRC screening that can also be applied as a robust evaluator of mucosal healing in UC patients.

A retrospective design and single-hospital dataset analyses are limitations of this study. However, we argue that the observational nature of the study, which did not require interventions in clinical practice, should limit bias in the results. Despite its limitations, our study revealed that the clinical prognosis of UC patients in remission differs between patients with complete endoscopic remission (MES 0) and those without (MES 1-3). We also demonstrate that in patients who are one year or more removed from UC remission induction, there is a strong positive correlation between negative FIT results, an MES of 0 and better prognosis. We suggest performing the noninvasive FIT in UC patients in prolonged remission (in place of endoscopy) to simplify the assessment of healing and meeting of treatment goals. These findings may greatly improve clinical practice in the evaluation of UC patients, particularly those in clinical remission.

COMMENTS

Background

Mucosal healing is a treatment goal for better prognosis in ulcerative colitis (UC). The authors previously reported that the quantitative fecal immunochemical test (FIT) effectively reflects the mucosal status of UC and that a negative FIT strongly correlates with mucosal healing.

Research frontiers

Currently, the definition of mucosal healing is not yet standardized. Some reports have defined mucosal healing with a Mayo endoscopic subscore (MES) of 0 or 1, whereas others define healing as only an MES of 0. The differences in the clinical outcomes between MES 0 and MES 1 patients have not yet been systematically evaluated. In addition, it has not yet been established whether FIT results can function as a surrogate marker for prognosis in UC patients in clinical remission.

Innovations and breakthroughs

Patients with an MES of 0 were much less likely to relapse than those with an MES of 1. Thus, an MES of 0 should be a treatment goal and an optimal definition of mucosal healing. In addition, this is the first study to demonstrate that FIT results can predict the prognosis of UC. In patients in clinical remission, those with a negative FIT result were less likely to relapse than patients with a positive FIT. Moreover, negative FIT results one year or more after remission induction correlated with an MES of 0 and better prognosis.

Applications

The endoscopic features of the appropriate treatment goal of UC were indicated. The predictability of the FIT as a measure of mucosal status and the prognosis of patients with clinical remission were shown. Patients with a negative FIT demonstrated a lower risk of clinical relapse than those with a positive FIT. Additional results revealed that the risk of relapse in patients in prolonged remission and with a negative FIT were similar to those with an MES of 0. Taken together, these findings would be useful in an economical follow-up of UC patients.

Peer-review

The manuscript by Asuka Nakarai and colleagues describe a retrospective cohort study on ulcerative colitis. This manuscript is prepared with care and detail. Significance of the study is reflected by analysing human data over time. Ethical data and information are provided. Authors declared no conflict of interest.

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P- Reviewer: Braet F, Hokama A **S- Editor**: Qi Y **L- Editor**: A
E- Editor: Ma S

World Journal of Gastroenterology

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Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.3748/wjg.v22.i21.5088

World J Gastroenterol 2016 June 7; 22(21): 5088-5095
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

ORIGINAL ARTICLE

Retrospective Study

Blood neutrophil-lymphocyte ratio predicts survival after hepatectomy for hepatocellular carcinoma: A propensity score-based analysis

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Supported by National Natural Science Foundation of China, No. 81260331; and the Key Laboratory for High-Incidence Tumor Prevention and Treatment, Ministry of Education, No. GKE2015-ZZ05.

Institutional review board statement: The study was reviewed and approved by the Tumor Hospital of Guangxi Medical University Institutional Review Board.

Informed consent statement: All study participants, or their legal guardian, provided informed written consent prior to study enrollment.

Conflict-of-interest statement: No potential conflicts of interest relevant to this article were reported.

Data sharing statement: Technical appendix, statistical code, and dataset are available from the corresponding author at cdsdyrmyy01@163.com. Participants gave informed consent for data sharing.

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Received: February 2, 2016
Peer-review started: February 2, 2016
First decision: March 7, 2016
Revised: March 22, 2016
Accepted: April 7, 2016
Article in press: April 7, 2016
Published online: June 7, 2016

Abstract

AIM: To investigate whether an elevated preoperative neutrophil-to-lymphocyte ratio (NLR) can predict poor survival in patients with hepatocellular carcinoma (HCC).

METHODS: We retrospectively reviewed 526 patients with HCC who underwent surgery between 2004 and

2011.

RESULTS: Preoperative NLR ⩾ 2.81 was an independent predictor of poor disease-free survival (DFS, $P < 0.001$) and overall survival (OS, $P = 0.044$). Compared with patients who showed a preoperative NLR < 2.81 and postoperative increase, patients who showed preoperative NLR ⩾ 2.81 and postoperative decrease had worse survival (DFS, $P < 0.001$; OS, $P < 0.001$). Among patients with preoperative NLR ⩾ 2.81, survival was significantly higher among those showing a postoperative decrease in NLR than among those showing an increase (DFS, $P < 0.001$; OS, $P < 0.001$). When elevated, alpha-fetoprotein (AFP) provided no prognostic information, and so preoperative NLR ⩾ 2.81 may be a good complementary indicator of poor OS whenever AFP levels are low or high.

CONCLUSION: Preoperative NLR ⩾ 2.81 may be an indicator of poor DFS and OS in patients with HCC undergoing surgery. Preoperative NLR ⩾ 2.81 may be a good complementary indicator of poor OS when elevated AFP levels provide no prognostic information.

Key words: Blood neutrophil-to-lymphocyte ratio; Hepatocellular carcinoma; Liver resection; Prognosis; Postoperative change in neutrophil-to-lymphocyte ratio

Core tip: We retrospectively analyzed a relatively large cohort of patients and used propensity score matching to balance out biases related to patient selection. Our results suggest that preoperative neutrophil-to-lymphocyte ratio (NLR) is a significant predictor of poor overall and disease-free survival. We further suggest that postoperative decrease in NLR is associated with poor survival, although only in patients with high preoperative NLR. Finally, we show that preoperative NLR ⩾ 2.81 may be a good complementary indicator of poor overall survival when elevated alpha-fetoprotein levels provide no prognostic information.

Yang HJ, Guo Z, Yang YT, Jiang JH, Qi YP, Li JJ, Li LQ, Xiang BD. Blood neutrophil-lymphocyte ratio predicts survival after hepatectomy for hepatocellular carcinoma: A propensity score-based analysis. *World J Gastroenterol* 2016; 22(21): 5088-5095 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i21/5088.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i21.5088

INTRODUCTION

Hepatocellular carcinoma (HCC) is a devastating malignancy that is the third most frequent cause of cancer-associated mortality worldwide. Hepatectomy and transplantation are considered curative treatments for HCC, but long-term survival is far from satisfactory due to the high frequency of tumor recurrence[1,2].

The prognosis of HCC patients who undergo resection varies, as it is dependent on such factors as tumor size, tumor number, vascular invasion, and tumor capsule, but these factors can only be assessed after surgery and so cannot be used for preoperative patient selection. One potential preoperative prognostic indicator is the neutrophil-to-lymphocyte ratio (NLR). This indicator of systemic inflammation is easy and inexpensive to determine[3-7], and elevated pretreatment NLR has been associated with poor outcome in numerous malignancies, including colon cancer[4], gastric cancer[8], HCC[3], and breast cancer[6].

Although studies have suggested that an elevated pretreatment NLR may correlate with a poor outcome in patients with HCC[9-11], other studies failed to detect such a correlation[12-14]. To gain a clearer picture of the influence of preoperative NLR on survival and recurrence after surgery for HCC, we carried out a retrospective study on propensity score-matched patients.

NLR often changes after hepatic resection in HCC patients, perhaps reflecting the shifting balance between inflammatory activity and immune activity. This raises the question of whether a postoperative change in NLR also serves as a predictor of prognosis after surgery. A study of 189 patients with early stage HCC suggested that a postoperative increase in NLR was associated with poorer overall survival and disease-free survival[13], but this has yet to be confirmed in larger samples.

The present study retrospectively analyzed a relatively large sample of Chinese patients with HCC in order to assess the usefulness of both preoperative NLR and postoperative change in NLR as prognostic indicators.

MATERIALS AND METHODS

This research was approved by the Ethics Committee of the Tumor Hospital of Guangxi Medical University. Written informed consent was obtained from participating patients.

Patients

All patients who underwent hepatic resection for primary HCC as initial treatment at the Affiliated Tumor Hospital of Guangxi Medical University between May 2004 and September 2011 were considered for inclusion in the study. Patients were diagnosed with primary HCC when two types of imaging technique showed features typical of HCC, or when one imaging technique gave positive findings and the alpha fetoprotein (AFP) level was > 400 ng/mL. Diagnosis of HCC was confirmed by histopathological examination.

Patients were excluded from the study if they underwent transarterial chemoembolization (TACE),

radiofrequency ablation (RFA), percutaneous ethanol injection, or other anti-tumor therapies before hepatic resection. Patients were also excluded if they suffered from preoperative fever.

Baseline clinical characteristics and laboratory results were recovered from the hospital database.

Definition of NLR

NLR was calculated by dividing the neutrophil count by the lymphocyte count. Preoperative NLR was determined within 7 d of surgery, and postoperative NLR was determined at the first follow-up visit in the outpatient department a month after surgery. Postoperative change in NLR was calculated by dividing postoperative NLR by preoperative NLR. The resulting numerical changes were transformed into a binary outcome of postoperative increase in NLR (when the ratio was $\geqslant 1$) or postoperative decrease in NLR (when the ratio was < 1). For certain analyses, patients were divided into groups with low or high preoperative NLR using a cutoff value of 2.81, as reported in the literature[9,11].

Follow-up visits and outcomes

All patients were followed up 1 mo after liver resection, every subsequent 3 mo during the first postoperative year, and every 6 mo thereafter until 60 mo after surgery or until death. At each follow-up visit, routine blood tests, serum AFP assay, ultrasound and computed tomography (CT), or magnetic resonance imaging (MRI) were performed.

Outcomes were overall survival (OS) and disease-free survival (DFS). DFS was defined as the interval from hepatectomy to imaging-based discovery of tumor relapse. OS was defined as 60 mo for those who survived more than 60 mo and DFS was defined as 60 mo if tumor relapse did not occur within 60 mo.

Propensity score analysis

Since patients were assigned to groups based on a preoperative NLR cut-off rather than randomization, propensity score analysis was used to balance out patient differences related to patient selection for hepatic resection. Propensity scores for all patients were estimated using a logistic regression model, which included all covariates that might have affected patient assignment to a high or low preoperative NLR group, as well as patient survival (Table 1). One-to-one nearest-neighbor matching was performed between high and low preoperative NLR using a 0.1 caliper width[14]. The resulting score-matched pairs were used in subsequent analyses as indicated.

Statistical analysis

Statistical analysis was performed using SPSS 19.0 (IBM, United States). Intergroup differences in categorical data were assessed for significance using χ^2 test, while intergroup differences in continuous data were assessed using the Mann-Whitney *U* test or *t* test. OS and DFS were analyzed using the Kaplan-Meier approach, and differences were assessed for significance using the log-rank test. Independent prognostic factors were identified using the Cox proportional hazards model. $P < 0.05$ served as the threshold of significance.

RESULTS

Study population

Between May 2004 and September 2011, 858 patients underwent hepatectomy for HCC at the Affiliated Tumor Hospital of Guangxi Medical University. Of these, 332 (38.7%) were excluded from our study because they (1) received initial HCC treatment at other centers (n = 288, 33.5%); (2) had already undergone RFA, TACE, percutaneous ethanol injection, or another pre-resection procedure (n = 24, 2.8%); or (3) suffered from preoperative fever (n = 20, 2.3%).

Ultimately, 526 patients (61.5%) were enrolled in the study, of whom 452 (85.9%) received curative hepatectomy. The remaining 74 patients (14.1%) received hepatectomy that was considered palliative because they had macroscopic vessel invasion[15].

Clinicopathological characteristics

Of the 526 patients, 125 (23.8%) had NLR levels higher than the cut-off value and were included in the high NLR group, while the remaining 401 (76.2%) were included in the low NLR group. The two groups were balanced in terms of gender, age, Edmondson grade, surgical margin, Child-Pugh class, and tumor number, as well as levels of albumin, aspartate aminotransferase (AST), and alanine aminotransferase (ALT) (all $P > 0.05$; Table 1). However, the two groups were unbalanced in terms of the presence of hepatitis B surface antigen (HbsAg), liver cirrhosis, tumor capsule, and vascular invasion; levels of AFP, platelets, and total bilirubin; Barcelona Clinic Liver Cancer (BCLC) stage; and tumor size (all $P < 0.05$; Table 1). Propensity score matching was used to generate 111 pairs of patients from the two groups, who showed no significant differences (Table 1).

Survival among all patients and propensity-matched pairs

Among all patients in the study, DFS was 55.4% at 1 year, 37.3% at 3 years, and 19.6% at 5 years. The corresponding OS rates were 78.2%, 57.9%, and 35.6%, respectively. Among propensity-matched pairs of patients, DFS was significantly higher in the low-NLR group than in the high-NLR group at 1, 3, and 5 years (Figure 1A). Similar results were obtained for OS (Figure 1B).

Risk factors for prognosis after hepatectomy

Among all patients in the study, univariate analysis

Table 1 Clinicopathological variables in Chinese patients with hepatocellular carcinoma treated by hepatic resection

Variable	Before propensity matching			After propensity matching		
	NLR < 2.81 *n* = 401	NLR ⩾ 2.81 *n* = 125	*P* value	NLR < 2.81 *n* = 111	NLR ⩾ 2.81 *n* = 111	*P* value
Gender, M/F	358/43	107/18	0.262	94/17	97/14	0.561
Age (yr)	46.8 ± 10.9	47.9 ± 11.5	0.309	46.1 ± 11.6	47.2 ± 11.2	0.487
HbsAg						
Negative	50	25	0.041	18	19	0.857
Positive	351	100		93	92	
Liver cirrhosis						
Yes	338	94	0.023	85	86	0.873
No	63	31		26	25	
AFP (ng/mL)						
< 400	268	70	0.032	67	67	1.000
⩾ 400	133	55		44	44	
Edmonson grade						
Ⅰ-Ⅱ	238	84	0.166	71	71	1.000
Ⅲ-Ⅳ	163	41		40	40	
Surgical margin (cm)						
< 1	195	51	0.126	48	50	0.787
⩾ 1	206	74		63	61	
BCLC stage						
0 or A	180	69	0.044	53	57	0.591
B or C	221	56		58	54	
Child-Pugh class						
A	385	117	0.260	105	105	1.000
B	16	8		6	6	
Tumor number						
Single	293	86	0.363	75	79	0.560
Multiple	108	39		36	32	
Tumor size (cm)	6 (4-8)	8 (5.5-12)	< 0.001	7.5 (6-11)	8 (5-12)	0.769
Tumor capsule						
Complete	181	40	0.010	33	39	0.390
Incomplete	220	85		78	72	
Vascular invasion						
Absent	354	98	0.008	91	91	1.000
Present	47	27		20	20	
Albumin (g/L)	40.1 ± 4.4	39.8 ± 4.1	0.399	40.3 ± 5.1	39.9 ± 4.2	0.583
Platelet count (10^9/L)	180.6 ± 76.6	202.1 ± 84.7	0.008	206.5 ± 82.6	200.91 ± 85.73	0.624
AST (U/L)	41 (36-60)	49 (37-67.5)	0.371	47 (31-70)	49 (37-70)	0.138
ALT (U/L)	40 (29-58)	42 (27.5-54)	0.393	37 (26-59)	42 (33-55)	0.400
Total bilirubin (μmol/L)	12 (9-16.8)	14 (9.9-19.5)	0.010	12.3 (9.2-17.4)	14 (9.9-19.4)	0.051

Data are mean ± SD or median (25^{th}-75^{th} interquartile range) unless otherwise indicated. AFP: Alpha-fetoprotein; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; HBsAg: Hepatitis B surface antigen; NLR: Blood neutrophil-to-lymphocyte ratio.

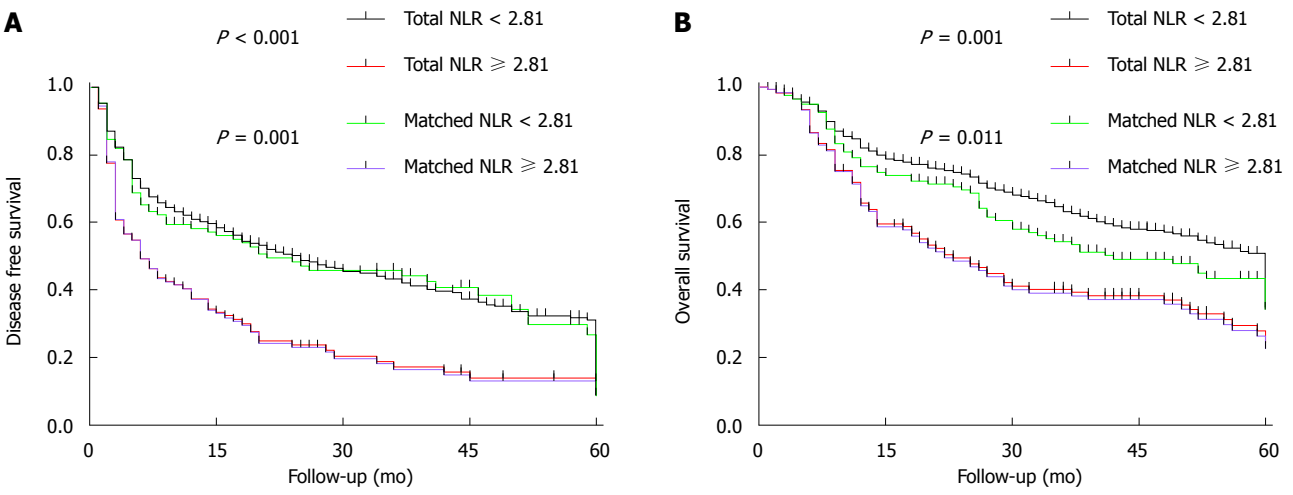

Figure 1 Post-hepatectomy disease-free survival (A) and overall survival (B) of hepatocellular carcinoma patients with high or low neutrophil-to-lymphocyte ratio. Separate curves are shown for the entire cohort (*n* = 526) and the propensity-matched cohort (*n* = 222). NLR: Neutrophil-to-lymphocyte ratio.

Preoperative NLR	Postoperative change in NLR	*n*	Disease-free survival, mo	*P* value
< 2.81	Decrease	227	32.3 ± 1.8	
< 2.81	Increase	124	33.7 ± 2.3	
⩾ 2.81	Decrease	92	20.9 ± 2.4	
⩾ 2.81	Increase	13	2.9 ± 0.5	
< 2.81	Decrease	227	44.6 ± 1.3	
< 2.81	Increase	124	47.7 ± 1.6	
⩾ 2.81	Decrease	92	37.1 ± 2.3	
⩾ 2.81	Increase	13	10.6 ± 2.6	

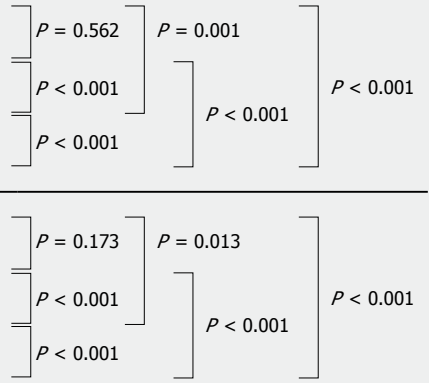

Figure 2 Comparison of survival of Chinese patients with hepatocellular carcinoma, stratified by preoperative neutrophil-to-lymphocyte ratio and postoperative change in neutrophil-to-lymphocyte ratio. NLR: Neutrophil-to-lymphocyte ratio.

Table 2 Multivariate analysis to identify factors predicting poor overall survival and disease-free survival in Chinese patients with hepatocellular carcinoma after hepatectomy

Factor	HR	95%CI	*P* value
Disease-free survival			
AFP > 400 ng/mL	1.493	1.062-2.100	< 0.001
Multiple tumors	1.766	1.385-2.252	< 0.001
Tumor size ⩾ 5 cm	1.313	1.018-1.693	0.036
Vascular invasion	2.656	1.962-3.594	< 0.001
NLR ⩾ 2.81	1.610	1.250-2.075	< 0.001
Overall survival outcome			
Multiple tumors	1.649	1.257-2.16	< 0.001
Tumor size ⩾ 5 cm	1.912	1.407-2.59	< 0.001
Incomplete tumor capsule	1.480	1.139-1.92	0.003
Vascular invasion	2.239	1.496-3.350	< 0.001
NLR ⩾ 2.81	1.333	1.007-1.76	0.044

Calculated over all patients in the study (n = 526). AFP: Alpha-fetoprotein.

identified several factors significantly associated with poor DFS: AFP ⩾ 400 ng/mL, Edmondson grade Ⅲ-Ⅳ, surgical margin < 1 cm, multiple tumors, tumor size ⩾ 5 cm, incomplete tumor capsule, vascular invasion, preoperative NLR ⩾ 2.81, AST ⩾ 80 U/L, and BCLC stage B or C. With the exception of AFP level, all of the aforementioned factors were also found to be significantly associated with poor OS.

Multivariate analysis (Table 2) identified the following independent predictors of poor DFS: AFP ⩾ 400 ng/mL, multiple tumors, tumor size ⩾ 5 cm, vascular invasion, and preoperative NLR ⩾ 2.81. Excluding AFP level, all of these factors were also found to be independent predictors of poor OS.

Postoperative change in NLR as possible prognostic factor

In the complete cohort of 526 patients, postoperative NLR data were available for 456 (86.7%). These fell into the following four subgroups (Figure 2): 227 patients (49.8%) who had a preoperative NLR < 2.81 and showed a postoperative decrease in NLR; 124 (27.2%) who had a preoperative NLR < 2.81 and showed a postoperative increase in NLR; 92 (20.2%) with preoperative NLR ⩾ 2.81 and a postoperative decrease in NLR; and 13 (2.9%) with NLR ⩾ 2.81 and a postoperative increase in NLR.

Compared with the patients who show preoperative NLR < 2.81 and postoperative increase, the patients who show preoperative NLR ⩾ 2.81 and postoperative decrease have worse survival (DFS, P < 0.001; OS, P < 0.001; Figure 2). Among patients with preoperative NLR ⩾ 2.81, survival was significantly higher for those showing a postoperative decrease in NLR than for those showing a postoperative increase (DFS, P < 0.001; OS, P < 0.001; Figure 2).

Prognostic value of preoperative NLR based on AFP levels

Since univariate analysis identified preoperative AFP ⩾ 400 ng/mL as a predictor of poor DFS but not OS, we wanted to examine whether the prognostic value of preoperative NLR varied with AFP level. Analysis of patient subgroups with AFP levels of 200, 400 ng/mL showed that, when elevated, AFP levels provide no prognostic information and that preoperative NLR ⩾ 2.81 may be a complementary indicator of poor OS whenever alpha-fetoprotein (AFP) levels are low or high (Figure 3).

DISCUSSION

The present retrospective study with a relatively large cohort of Chinese HCC patients suggests that elevated preoperative NLR is associated with poor OS and DFS, and that a postoperative increase in NLR is associated with poor survival. This result may not only assist surgeons in predicting HCC patient survival before and after surgery, but also act to remind the surgeon to perform timely adjuvant treatment to improve the

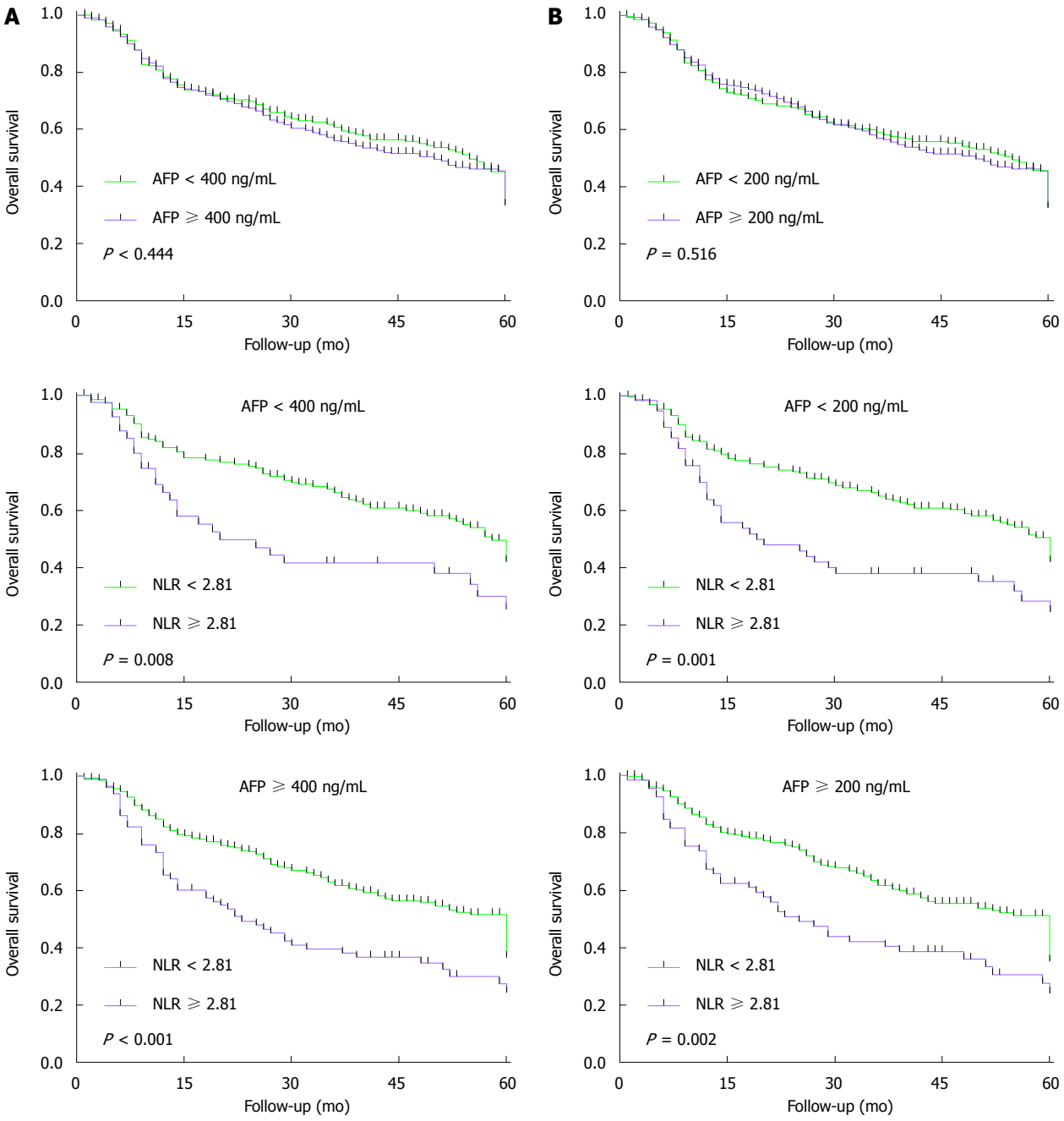

Figure 3 Comparison of overall survival of hepatocellular carcinoma patients stratified based on preoperative alpha-fetoprotein level and on high or low preoperative neutrophil-to-lymphocyte ratio. Patients were grouped using alpha-fetoprotein cut-off values of 400 ng/mL (A), 200 ng/mL (B). Results are shown only for the entire cohort (n = 526). NLR: Neutrophil-to-lymphocyte ratio.

prognosis of patients with preoperative NLR ⩾ 2.81.

In our cohort, elevated serum AFP levels were not significant predictors of poor OS after resection: OS did not vary significantly with preoperative AFP levels of 200, 400 ng/mL. AFP remains controversial as a predictor of HCC patient survival after resection; while some studies have associated elevated serum AFP levels with poor prognosis[11,16-18], others have failed to find such an association[10,13,15,19]. Our results suggest that, when elevated AFP levels provide no prognostic information, preoperative NLR ⩾ 2.81 may be a complementary indicator of poor OS whenever AFP levels are low or high.

Why elevated NLR and postoperative NLR increase should predict poor survival remains unclear, but some studies have proposed explanations. One such explanation is that many patients with elevated NLR have lymphocytopenia, which may contribute to a weak lymphocyte-mediated immune response to tumors[20]. This lymphocyte-mediated response normally aids in the elimination of abnormal cells and in the production of cytokines that inhibit tumor proliferation, invasion, and metastasis[21]. Another possible explanation is that elevated NLR reflects a stronger neutrophil response

and higher numbers of peripheral neutrophils, leading to higher secretion of pro-angiogenic factors such as interleukin-8[22], vascular endothelial growth factor (VEGF)[23,24], and matrix metalloproteinase (MMP)[25,26], which may contribute to tumor growth and therefore to poor prognosis. Studies have indicated that a postoperative NLR increase may reflect that the body has not recovered from tumor control after surgery[27], potentially leading to worse survival.

The findings of the present study should be interpreted with caution in light of several limitations. First, this study is retrospective and based on patients at a single institution. Indeed, more than 85% of our cohort was chronically infected with hepatitis B virus, which is not the case in other parts of the world. Secondly, the cut-off value of NLR was obtained from published papers[9,11]. Thirdly, owing to the distribution of HCC patients, the number of patients who showed preoperative NLR ⩾ 2.81 and postoperative increase was more than 10 times less than others, which may have led to variance in the results.

In conclusion, our results suggest that both preoperative NLR and postoperative change in NLR are predictors of OS and DFS in HCC patients undergoing hepatic surgery. Elevated NLR may be a complementary, or even an alternative, biomarker of survival when elevated AFP levels prove uninformative.

ACKNOWLEDGMENTS

The authors thank Armando Chapin Rodríguez, PhD, for his language editing, which substantially improved the quality of the manuscript.

COMMENTS

Background

An elevated preoperative neutrophil-to-lymphocyte ratio (NLR) may predict poor survival in patients with hepatocellular carcinoma (HCC), but this requires confirmation.

Research frontiers

The prognosis of HCC patients who undergo resection depends on factors such as tumor size and number, vascular invasion, and tumor capsule, but these factors can only be assessed after surgery and so cannot be used for preoperative patient selection. One potential preoperative prognostic indicator is the neutrophil-to-lymphocyte ratio. This indicator of systemic inflammation is easy and inexpensive to determine.

Innovations and breakthroughs

The authors retrospectively analyzed a relatively large cohort of patients and used propensity score matching to balance out biases related to patient selection in order to investigate the impact of preoperative NLR and postoperative NLR on survival. This study will provide more evidence for NLR after curative resection of HCC in the future.

Applications

Preoperative NLR ⩾ 2.81 may be an indicator of poor DFS and OS in patients with HCC undergoing surgery. Preoperative NLR ⩾ 2.81 may be a good complementary indicator of poor OS when elevated AFP levels provide no prognostic information.

Terminology

NLR was calculated by dividing the neutrophil count by the lymphocyte count. Postoperative change in NLR was calculated by dividing postoperative NLR by preoperative NLR. The resulting numerical changes were transformed into a binary outcome of postoperative increase in NLR (when the ratio was ⩾ 1) or postoperative decrease in NLR (when the ratio was < 1).

Peer-review

This is an interesting manuscript that shows the benefit of using an easily available tool for prognosis after resection for HCC.

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P- Reviewer: Kapoor S, Morales-Gonzalez J **S- Editor**: Qi Y
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DOI: 10.3748/wjg.v22.i21.5096

World J Gastroenterol 2016 June 7; 22(21): 5096-5103
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

ORIGINAL ARTICLE

Observational Study

Serum adipokines might predict liver histology findings in non-alcoholic fatty liver disease

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Author contributions: Jamali R and Aarabi MH proposed the idea and designed the research; Jamali R, Arj A and Razavizade M diagnosed NAFLD and enrolled patients; Jamali R, Arj A, Aarabi MH and Razavizade M collected the data; Jamali R performed the statistical analysis and interpreted the data; Jamali R and Aarabi MH wrote the draft; all authors read and approved the final manuscript.

Institutional review board statement: The study was reviewed and approved by the Kashan University of Medical Sciences Institutional Review Board.

Informed consent statement: All study participants, or their legal guardians, provided informed written consent before study enrollment.

Conflict-of-interest statement: No potential conflicts of interest relevant to this article were reported.

Data sharing statement: No additional data are available.

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Received: January 5, 2016
Peer-review started: January 5, 2016
First decision: February 18, 2016
Revised: February 25, 2016
Accepted: March 18, 2016
Article in press: March 18, 2016
Published online: June 7, 2016

Abstract

AIM: To assess significance of serum adipokines to determine the histological severity of non-alcoholic fatty liver disease.

METHODS: Patients with persistent elevation in serum aminotransferase levels and well-defined characteristics of fatty liver at ultrasound were enrolled. Individuals with a history of alcohol consumption, hepatotoxic medication, viral hepatitis or known liver disease were excluded. Liver biopsy was performed to confirm non-alcoholic liver disease (NAFLD). The degrees of liver steatosis, lobular inflammation and fibrosis were determined based on the non-alcoholic fatty liver activity score (NAS) by a single expert pathologist. Patients with a NAS of five or higher were considered to have steatohepatitis. Those with a NAS of two or lower were defined as simple fatty liver. Binary logistic regression was used to determine the independent association of adipokines with histological findings. Receiver operating characteristic (ROC) analysis was employed to determine cut-off values of serum adipokines to discriminate the grades of liver steatosis,

lobular inflammation and fibrosis.

RESULTS: Fifty-four participants aged 37.02 ± 9.82 were enrolled in the study. Higher serum levels of visfatin, IL-8, TNF-α levels were associated independently with steatosis grade of more than 33% [β = 1.08 (95%CI: 1.03-1.14), 1.04 (95%CI: 1.008-1.07), 1.04 (95%CI: 1.004-1.08), $P < 0.05$]. Elevated serum IL-6 and IL-8 levels were associated independently with advanced lobular inflammation [β = 1.4 (95%CI: 1.09-1.8), 1.07 (95%CI: 1.003-1.15), $P < 0.05$]. Similarly, higher TNF-α, resistin, and hepcidin levels were associated independently with advanced fibrosis stage [β = 1.06 (95%CI: 1.002-1.12), 19.86 (95%CI: 2.79-141.19), 560.72 (95%CI: 5.98-5255.33), $P < 0.05$]. Serum IL-8 and TNF-α values were associated independently with the NAS score, considering a NAS score of 5 as the reference value [β = 1.05 (95%CI: 1.01-1.1), 1.13 (95%CI: 1.04-1.22), $P < 0.05$].

CONCLUSION: Certain adipokines may determine the severity of NAFLD histology accurately.

Key words: Non-alcoholic fatty liver disease; Adipokine; Histology; Adiponectin; Visfatin; Resistin; Hepcidin; Interleukin; Tumor necrosis factor

Core tip: Considering the drawbacks of current assays, it seemed reasonable to find appropriate serum biomarkers to define the extent of liver damage in non-alcoholic liver disease (NAFLD). We investigated several key adipokines together with metabolic profiles and liver function tests, providing an advantage over previous studies. We concluded that serum visfatin, IL-8, TNF-α levels were associated with liver steatosis degree; serum IL-6 and IL-8 concentrations correlated with lobular inflammation grade; and TNF-α, resistin, and hepcidin levels correlated with fibrosis stage. The study suggested that certain adipokines might have better accuracy than currently used serum biomarkers to determine NAFLD histology.

Jamali R, Razavizade M, Arj A, Aarabi MH. Serum adipokines might predict liver histology findings in non-alcoholic fatty liver disease. *World J Gastroenterol* 2016; 22(21): 5096-5103 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i21/5096.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i21.5096

INTRODUCTION

Non-alcoholic liver disease (NAFLD) is a health concern worldwide. The burden of the disease is increasing because of the epidemic of obesity and the development of insulin resistance (IR) syndrome[1]. Liver function tests, metabolic profiles, liver ultrasound and clinical data are used routinely to detect the disease. Considering the limitations of these assays, liver biopsy is still the gold standard method to diagnose NAFLD[2]. However, concerns about the possible complications and invasiveness of the method have limited its application by physicians. It seems reasonable to identify appropriate serum biomarkers to diagnose and define the extent of liver damage in NAFLD. In this regard, interest in the roles of adipokines that are secreted from visceral adipose tissue (VAT) has been increasing.

NAFLD comprises a wide spectrum of liver cell injury that is induced by insulin resistance. Primarily, the accumulation of fat occurs in hepatocytes (simple fatty liver) as a consequence of hepatic insulin resistance. A growing body of evidence supports the view that adipokines modulate these metabolic processes by regulating insulin mediated glucose metabolism, fatty acid utilization and lipid accumulation of visceral tissues. At the later stages of disease, inflammatory phenomena arise that might progress to steatohepatitis and, ultimately, cirrhosis. It has been suggested that the development of steatohepatitis is a consequence of the balance between pro and anti-inflammatory effects of adipokines.

There is a paucity of literature regarding the serum threshold values and efficacy of adipokines in the diagnosis and follow-up of fatty liver patients. In this research, we evaluated certain important adipokines that were reported to be associated with NAFLD, in a cohort of biopsy-proven NAFLD patients[3-9].

The aims of this study were: (1) to evaluate the association of histological findings (steatosis, lobular inflammation and fibrosis) and serum biomarkers (including adipokines, inflammatory cytokines, liver function tests and metabolic profiles); and (2) to determine cut-off values of serum biomarkers to identify the grades of steatosis, lobular inflammation and fibrosis.

MATERIALS AND METHODS

Patient enrolment protocol

This study was conducted in the outpatient gastroenterology clinic of Shahid Beheshti general hospital, from September 2012 to September 2014. Initially, patients with persistent elevated serum aminotransferase levels and well-defined characteristics of a fatty liver *via* abdominal ultrasound (Hitachi EUB 405 apparatus equipped with a convex 3.5 MHz probe) were included (Phase 1)[1,10]. The upper normal limit of the serum aminotransferases level was considered as 40 units per liter[11]. Individuals with a history of alcohol consumption, hepatotoxic medication, viral hepatitis and known liver disease were excluded from the study (Phase 2)[1,12]. Liver biopsy was performed on the remaining patients from phase 2 to confirm diagnosis of NAFLD for final enrolment (Phase 3).

Ethical considerations

The study was conducted according to ethical standards for human experimentation (Helsinki Declaration). The ethics committee of the hospital approved the study protocol (No: 8861). The purpose of study was explained to the participants. They were enrolled in the study upon providing written informed consent.

Sample size calculation

The sample size was n = 54, considering the mean prevalence of NAFLD (P = 28%, α = 0.05, z = 1.96, and d = 0.12), according to a previous study[10].

Laboratory assays

Fasting serum samples were obtained to assess the level of adiponectin, visfatin, resistin, hepcidin, IL-6, IL-8 and tumor necrosis factor (TNF)-α by enzyme-linked immunosorbent assay (ELISA), according to the manufacturer's instructions. The following kits were used in this study: Human adiponectin and visfatin ELISA kits (Production numbers: AG-45A-0001 and AG-45A-0006 respectively; ADIPOGEN Inc., South Korea), resistin (human resistin ELISA kit, Biovendor, Czech Republic), hepcidin (Lot: RN- 24429; DEMEDITEC GmbH, Kiel-Wellsee, Germany), IL-6 (Lot: 233737; Bendered Systems GmbH, Vienna, Austria), IL-8 (Lot: ab46032; IL-8 human ELISA kit, Abcam, United States), and TNF-α (Lot: ab46087; TNF-α human ELISA kit, Abcam, United States). Fasting blood glucose, insulin, lipid profiles and liver function tests were performed as previously described[1,10-13].

Liver histology

Percutaneous liver biopsy was performed using a true cut needle (G14). A sample larger than 10 mm or with at least five portal tracts was considered acceptable for histological evaluation. Hematoxylin and Eosin (HE) and Masson's Trichrome stainings were performed to evaluate necroinflammation and fibrosis, respectively. To avoid inter-observer disagreement, a single expert pathologist who was blinded to the patient data interpreted samples. The degree of liver steatosis, lobular inflammation and fibrosis was defined based on the "non-alcoholic fatty liver activity score (NAS)"[14]. Patients with a NAS of five or higher were considered to have NASH. Those with a NAS of two or lower were defined as simple fatty liver[14].

Statistical analysis

Continuous variables were reported as the mean ± SD and categorical variables were shown as counts (percent). The Kolmogorov-Smirnov test was used to assess the distribution of serum adipokines. A χ^2 or *t*-test was applied to assess differences among groups, where appropriate. Binary logistic regression analysis using the standard model was applied to evaluate the association of independent variables (including serum adipokines and clinical data) and liver histology findings.

Hepatic steatosis severity was categorized into four degrees according to the NAS. The first two degrees (0-1) represented no and mild liver steatosis, and the next degrees (2-3) indicated moderate to severe liver steatosis. To define the risk of lower liver steatosis versus a more advanced degrees of steatosis, we considered the patients with steatosis grades of less than 33% as the "mild group". Meanwhile, those with higher degrees (2-3) were merged to form the "moderate to severe group".

The lobular inflammation range was graded from 0 to 3 by the NAS. To estimate the risk of lower lobular inflammation against more advanced grades, we labeled the individuals with lobular inflammation of less than two foci per HPF (grade 1) as the "mild group". At the same time, those with higher lobular inflammation grades (2-3) were combined to form the "moderate to severe group".

Hepatic fibrosis content was categorized into 5 stages based on NAS. The former two stages (0-1) demonstrated none/mild fibrosis and the latter stages stand for more advanced fibrosis (2-4). In order to determine the probability of lower fibrosis versus more advanced fibrosis, we labeled the subjects with perisinusoidal or periportal (stage 1) as the "mild group". Those with higher fibrosis stages (2-4) were mixed to form the "moderate to severe group".

For the regression model, liver steatosis, lobular inflammation, fibrosis stage, and NAS were employed as dependent variables; Steatosis grade of less than 33%, lobular inflammation of less than two foci per high powered field (HPF), fibrosis stage of one (perisinusoidal or periportal), and a NAS of five or higher were set as the reference groups, respectively. Standardized correlation coefficient (OR) with the 95%CI was calculated. Serum adipokines that were independently associated with the histological findings were selected for receiver operating characteristic (ROC) analysis. ROC analysis explored the serum adipokines' cut-off values and their sensitivities and specificities to discriminate higher grades of liver steatosis, lobular inflammation and fibrosis. Values with the highest sum of the sensitivity and specificity were reported as the best cut-off values. All statistical analyses were performed by SPSS, version 17 (SPSS, Chicago, United States). The probability of a difference between groups was considered statistically significant if the two-sided P value was less than 0.05.

RESULTS

Seventy participants presumed to have NAFLD were evaluated from September 2012 to September 2014 (phase 1). Reasons for leaving certain patients out of the study were patient refusal to participate in the study (n = 8), normalization of ALT during the lead-in phase (n = 6), autoimmune hepatitis (n = 1) and viral

Table 1 Clinico-demographic and laboratory data of the participants

Variable	Total	Simple fatty liver	Non-alcoholic steatohepatitis
	n = 54	*n* = 2	*n* = 28
Age (yr)	37.02 ± 9.82	27.00 ± 2.82	35.00 ± 8.47
Male gender, *n* (%)	35 (64.8)	2 (100)	17 (60.7)
Waist circumference (cm)	102.13 ± 2.69	101.00 ± 42.24	101.57 ± 2.71
Body mass index (kg/m^2)	30.55 ± 3.97	28.09 ± 7.77	29.92 ± 3.79
Diabetes mellitus present, *n* (%)	12 (22.2)	0 (100)	11 (39.3)
Metabolic syndrome present, *n* (%)	36 (66.7)	1 (50)	21 (75)
Adiponectin (mg/L)	8.14 ± 2.91	8.20 ± 2.67	7.00 ± 0.28
Visfatin (ng/mL)	19.96 ± 17.5	5.40 ± 0.84	18.34 ± 16.18
Resistin (mg/mL)	2.51 ± 1.08	1.70 ± 1.83	2.10 ± 1.04
Hepcidin (ng/mL)	64.0 ± 0.62	48.50 ± 0.38	75.00 ± 0.49
Tumor necrosis factor-alpha (pg/mL)	2.68 ± 19.32	0.96 ± 9.05	3.68 ± 20.93
Interleukin 6 (pg/mL)	7.59 ± 5.75	4.70 ± 0.24	7.41 ± 4.78
Interleukin 8 (pg/mL)	27.41 ± 24.99	13.60 ± 13.01	38.60 ± 28.21
Alanine aminotransferase (U/L)	65.91 ± 36.11	37.00 ± 0.00	82.10 ± 39.25
Aspartate aminotransferase (U/L)	42.18 ± 20.48	25.00 ± 4.24	49.67 ± 24.14
Alkaline phosphatase (U/L)	181.50 ± 76.14	144.21 ± 42.41	180.23 ± 45.22
Gamma glutamyl transpeptidase (U/L)	54.12 ± 62.55	44.40 ± 30.54	55.40 ± 33.26
Fasting blood sugar (mg/dL)	98.41 ± 14.12	98.25 ± 16.31	103.0 ± 0.00
Insulin (mU/L)	15.16 ± 13.42	10.68 ± 3.65	17.40 ± 18.00
Triglyceride (mg/dL)	150.09 ± 70.18	60.20 ± 9.70	167.57 ± 77.19
Total cholesterol (mg/dL)	177.55 ± 34.17	165.85 ± 40.65	183.77 ± 37.76
Low density lipoprotein cholesterol (mg/dL)	100.89 ± 29.03	98.75 ± 35.42	103.15 ± 32.19
High density lipoprotein cholesterol (mg/dL)	48.51 ± 9.06	55.05 ± 7.14	48.66 ± 9.67

Data are presented as mean ± SD unless otherwise noted. Patients with non-alcoholic activity score (NAS) of five or higher were considered to have non-alcoholic steatohepatitis. Those with NAS equal to two or lower were defined as simple fatty liver[14]. NAFLD: Non-alcoholic fatty liver disease.

hepatitis (*n* = 1) (phase 2). Finally, fifty-four patients with biopsy proven NAFLD were included in the study (phase 3). The clinico-demographic and laboratory data of the participants are presented in Table 1.

Participants showed NAS of 4.87 ± 1.71. The frequency of histological findings in the study population is depicted in Figure 1.

Binary logistic regression showed a positive association between serum visfatin, IL-8 and TNF-α level and the grades of steatosis. Similarly, serum IL-6 and IL-8 levels were independently associated with the degrees of lobular inflammation. Serum TNF-α, resistin and hepcidin levels were independently associated with perisinusoidal fibrosis stage. Serum IL-8 and TNF-α values were positively associated with NAS (Table 2).

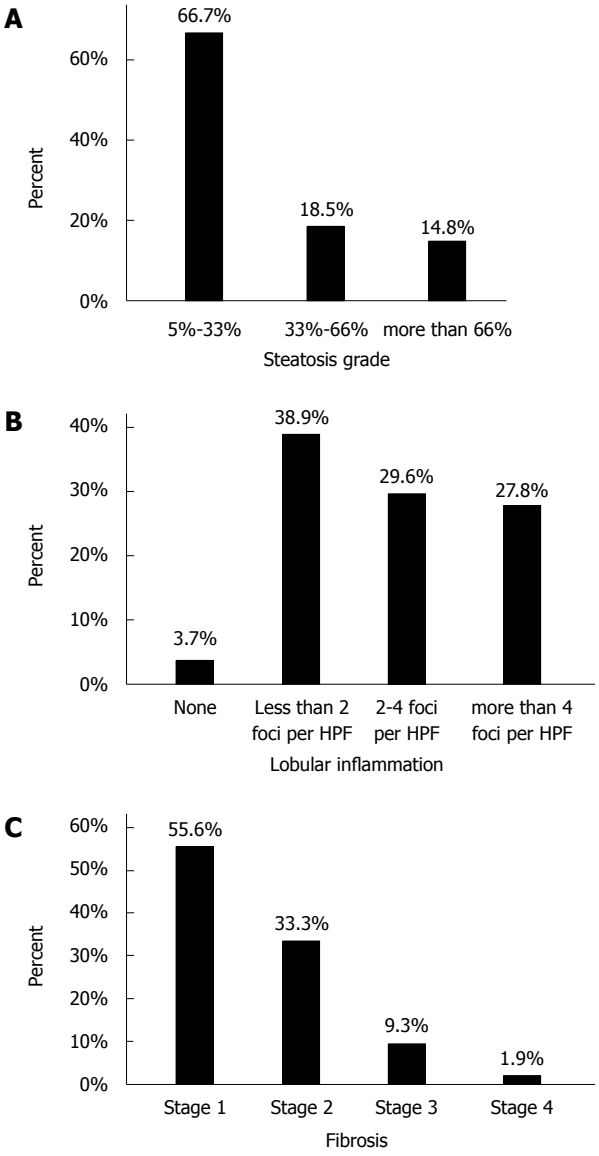

Figure 1 Frequency of histological findings in the participants. Frequency of patients with different degrees of steatosis (A), lobular inflammation grade based on foci of lobular inflammation in high power field of microscopic view (B), and fibrosis stage (C) are presented.

The ROC curves with calculated AUC (± 95%CI) to determine the best cut-off values of serum adipokines to differentiate histological groups are shown in Figure 2. The sensitivities and specificities of the cut-off values of biomarkers to identify histological groups appear in Table 3.

DISCUSSION

This study concluded that serum visfatin, IL-8 and TNF-α levels were independently associated with liver steatosis degree; serum IL-6 and IL-8 concentrations were independently associated with lobular inflammation grade; and TNF-α, resistin and hepcidin levels were independently associated with fibrosis stage in a cohort of biopsy-proven NAFLD patients. Moreover, the best cut-off values for the above-mentioned

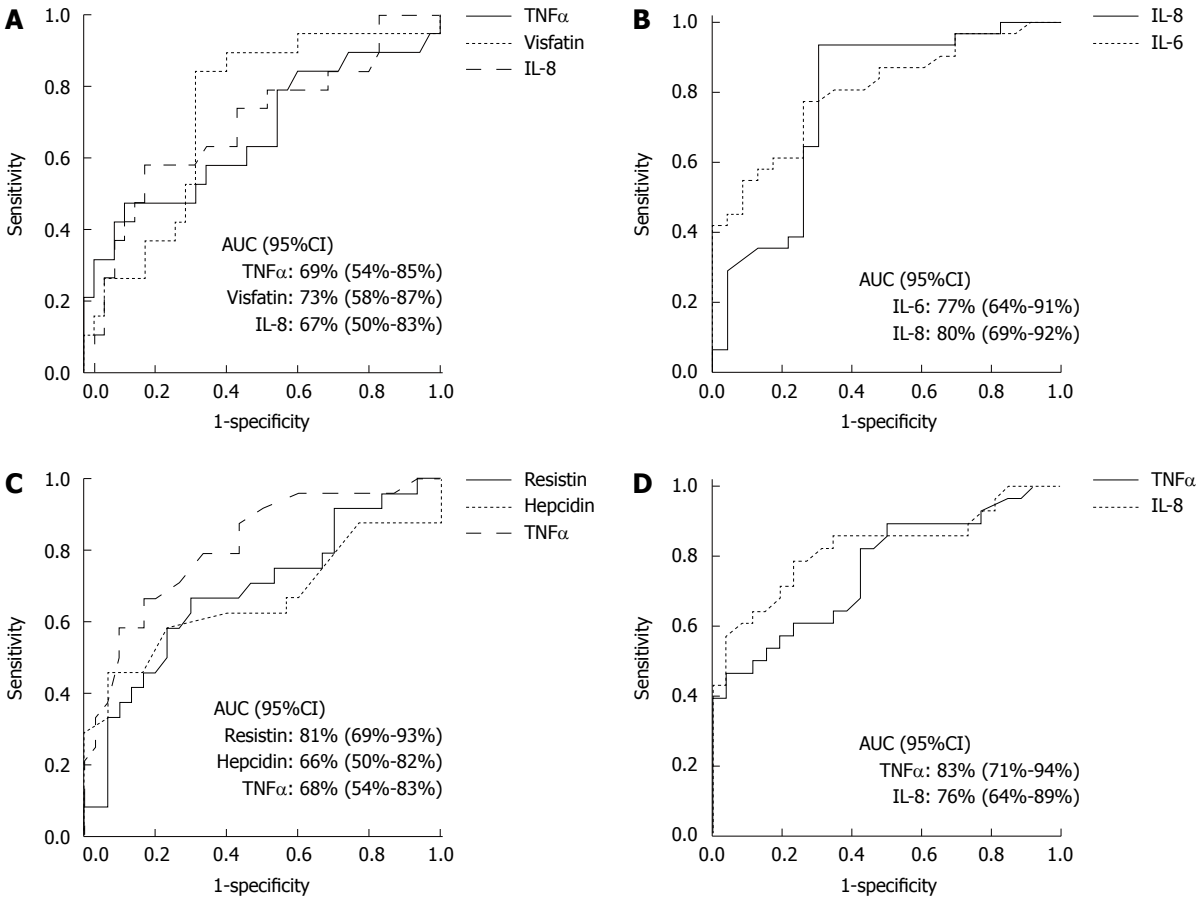

Figure 2 Receiver operating characteristic analysis to determine cut-off values of serum adipokines for differentiating histological severity. A: ROC curve of serum TNF-α, Visfatin, and IL-8 levels to differentiate steatosis degree of less than 33% from more advanced degrees of steatosis; B: ROC curve of serum IL-6 and IL-8 levels to differentiate lobular inflammation grade of less than two foci per high power field from more advanced grades of inflammation; C: ROC curve of serum Resistin, Hepcidin, and TNF-α levels to differentiate fibrosis stage of perisinusoidal or periportal from more advanced stages of fibrosis; D: ROC curve of serum TNF-α and IL-8 levels to differentiate steatohepatitis from simple fatty liver based on non-alcoholic fatty liver disease activity score. AUC: Area under curve; IL: Interleukin.

Table 2 Association between histological findings and serum adipokine levels

Adipokine	OR	95%CI	*P* value
Steatosis degree			
Visfatin	1.08	1.030-1.14	0.001
TNF-α	1.04	1.004-1.08	0.030
Interleukin 8	1.04	1.006-1.07	0.020
Lobular inflammation grade			
Interleukin 6	1.4	1.090-1.80	0.008
Interleukin 8	1.07	1.003-1.15	0.040
Fibrosis stage			
Resistin	19.86	2.790-141.19	0.003
Hepcidin	560.72	5.980-5255.33	0.006
TNF-α	1.06	1.002-1.12	0.040
NAS			
Interleukin 8	1.05	1.01-1.10	0.040
TNF-α	1.13	1.04-1.22	0.004

TNF-α: Tumor necrosis factor-alpha; NAS: Non-alcoholic fatty liver activity score.

serum adipokines were calculated to identify the liver histological findings.

The associations between certain adipokines with NAFLD were evaluated in previous reports[3-9]. We investigated several key adipokines, together with metabolic profiles and LFT, which provided an advantage over the previous studies. To improve the accuracy of the study, the cases were recruited from a cohort of biopsy-proven NAFLD patients. We used NAS for to determine the severity of the liver histology. Notably, NAS is a valid scoring system for NAFLD that differentiates the spectrum of disease with an acceptable reliability and validity[14].

Visfatin is a new adipokine with proinflammatory and metabolic properties. It is increased in IR syndrome. The expression of visfatin in VAT facilitates the maturation of preadipocyte cells to differentiated adipocytes (Paracrine effect)[3]. This fact might explain the correlation between serum visfatin levels and hepatic steatosis degree in our study. Visfatin is also associated with body fat mass in alcoholic fatty liver disease[15]. Previous studies have reported a correlation between visfatin and fibrosis stage, but not with steatosis or lobular inflammation grade in NAFLD[16]. Meanwhile, an increase in serum visfatin was shown to be associated with portal inflammation[17].

TNF-α is a pro-inflammatory cytokine and is asso-

Table 3 Best cut-off values of serum adipokine levels to differentiate histological groups according to receiver operating characteristic analysis

Adipokine	Serum concentration	Sensitivity (%)	Specificity (%)
TNF-α (pg/mL)	2.13	74	58
Visfatin (ng/mL)	13.00	84	69
Interleukin 8 (pg/mL)	24.25	58	66
Cut-off values of serum adipokine levels to differentiate lobular inflammation grade of less than 2 foci per high power field from more advanced grades of inflammation.			
Interleukin 6 (pg/mL)	3.70	94	70
Interleukin 8 (pg/mL)	13.00	77	74
Cut-off values of serum adipokine levels to differentiate perisinusoidal or periportal fibrosis from more advanced stages of fibrosis.			
Resistin (mg/mL)	1.65	79	66
Hepcidin (ng/mL)	45.00	63	60
TNF-α (pg/mL)	2.44	67	70
Cut-off values of serum adipokine levels to differentiate steatohepatitis from simple fatty liver group based on non-alcoholic fatty liver disease activity score (NAS).			
Interleukin 8 (pg/mL)	9.80	82	54
TNF-α (pg/mL)	2.44	71	81
Cut-off values of serum adipokine levels to differentiate steatosis degree of less than 33% from more advanced degrees of steatosis.			

TNF-α: Tumor necrosis factor-alpha.

ciated with hepatic IR in NAFLD[4]. It mediates the early stage of NAFLD by fat accumulation in hepatocytes. In addition, it facilitates disease progression to a more advanced stage[18]. The relationship between serum TNF-α and liver steatosis and fibrosis in our research is in line with previous observations.

IL-8 is also a pro-inflammatory cytokine that activate monocytes and attracts polymorphonuclear leukocytes to the site of inflammation[19]. It is increased in obese individuals with IR. In accordance with the literature, our results showed that serum IL-8 was associated with steatosis degree and lobular inflammation[5].

IL-6 is a liver and adipose tissue-derived proinflammatory cytokine that is implicated in hepatic and skeletal muscle IR. IL-6 is thought to act as a second hit in the pathophysiology of NAFLD, causing the progression of simple fatty liver to NASH[19]. The correlation of IL-6 with lobular inflammation grade in our study is comparable to the findings by other groups[6,20,21].

With regard to hepcidin, the circulatory level was strongly associated with fibrosis stage in our study. Nevertheless, a previous found no correlation between hepcidin and histological findings[7]. Body iron stores in NAFLD regulate hepcidin levels[22]. Therefore, it seems reasonable to adjust for patients iron storage when evaluating hepcidin levels in NAFLD patients.

Resistin is an adipokine that is considered an indicator of IR in obesity[23]. However, the pathophysiological role of resistin in NAFLD is not clear. In this study, we observed that serum resistin levels were related with fibrosis stage. On the other hand, advanced liver fibrosis was associated with reduced resistin concentration in chronic hepatitis C patients with normal body weight, glucose and lipid profiles[24]. Previous studies demonstrated a correlation between high serum resistin levels and the presence of steatosis and necroinflammation in NAFLD[8,25]. Meanwhile, another study demonstrated an association of low serum resistin levels with excessive fat accumulation in the liver[26].

Adiponectin is a well-known adipokine that regulates hepatic IR[27]. It was suggested that adiponectin might be related to steatosis grade and the severity of NAFLD; however, its definitive role remains to be addressed[28]. A decrease in serum adiponectin is the primary event in children with NAFLD before the rise of inflammatory cytokines and the development of overt diabetes[9,29]. One previous study showed that adiponectin could predict patients with higher necroinflammatory grade and fibrosis stage from those with milder histological findings[30]. Another study showed that adiponectin is related to hepatic fat content and not to necroinflammatory activity and fibrosis stage[31]. Meanwhile, our study showed no correlation between adiponectin and liver histology. This study, despite its advantages, suffers from several drawbacks: first, the study was performed in a single institution; therefore, the findings need to be generalized with caution. Second, our study was cross-sectional, which limited the interpretation of causal associations.

There is currently no defined "normal range" for serum adipokines. Moreover, adipokine levels might fluctuate over time according to the metabolic environment. These concerns might explain the differences in the results of the above-mentioned studies with our results. Further well-controlled prospective studies to determine the association of VAT-derived proteins (including proinflammatory cytokines and polypeptide hormones) and liver histological findings are recommended.

The associations of some important adipokines, together with the currently used serum biomarkers, with the liver histological findings were evaluated. Certain adipokines were independently associated with the liver histological findings. Finally, the best cut-off values of these serum adipokines were determined to detect the severity of liver steatosis, lobular inflammation and fibrosis.

In conclusion, this study suggested that certain adipokines might determine accurately the severity of NAFLD based on histological findings.

ACKNOWLEDGMENTS

We are grateful to Dr. Hassan Ehteram at the Kashan University of Medical Sciences for reviewing the liver histology samples, and to Dr. Ayat Ahmadi at the Tehran University of Medical Sciences (TUMS) for reviewing the statistical analysis. The authors would like to expand their gratitude to Drs. Vafa Rahimi

Movaghar and Arsia Jamali at TUMS for their critical review of the manuscript.

COMMENTS

Background

Non-alcoholic liver disease (NAFLD) is a health concern worldwide. The burden of disease is increasing because of an epidemic of obesity. Considering the limitations of current modalities, finding an appropriate serum biomarker to diagnose and assess the severity of liver damage in NAFLD is crucial.

Research frontiers

The roles of adipokines in the pathogenesis of NAFLD have received research interest recently. Nevertheless, there is a paucity of studies that used serum levels of adipokines in the diagnosis and follow up of NAFLD patients.

Innovations and breakthroughs

The associations between certain adipokines with NAFLD were evaluated in previous reports. The authors investigated several key adipokines together with metabolic profiles and LFT, providing an advantage over the previous studies. To improve the accuracy of the study, the cases were selected from a cohort of biopsy-proven NAFLD patients. To assess the severity of NAFLD based on histology, we applied NAS, a valid scoring system for NAFLD that categorizes the spectrum of disease with acceptable reliability and validity.

Applications

This study suggested that certain adipokines might determine accurately the presence and severity of NAFLD.

Peer-review

This manuscript evaluated the association between histological grade of NAFLD and serum biomarkers, and suggested cut-off values of the biomarkers for NASH. The authors used a direct method to define NASH, and measured various biomarkers related to adiposity and inflammation. Finally, the authors showed successfully that the indices were related to each component of NASH.

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World J Gastroenterol 2016 June 7; 22(21): 5104-5113
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

ORIGINAL ARTICLE

Prospective Study

Usefulness of portal vein pressure for predicting the effects of tolvaptan in cirrhotic patients

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Author contributions: Nakagawa A, Atsukawa M, Kondo C, Okubo T, Arai T and Itokawa N designed the study and measured porta vein pressure; Atsukawa M and Tsubota A contributed equally to the preparation of this manuscript; Tsubota A, Narahara Y and Iwakiri K supervised the study; all authors have read and approved the final version to be published.

Institutional review board statement: The protocol was approved by the Ethics Review Board of Nippon Medical School Chiba Hokusoh Hospital (approval No. 526012). All patients and their families received a sufficient explanation of the aim and contents of this study before the entry.

Conflict-of-interest statement: The authors declare that they have no conflict of interest.

Informed consent statement: All patients and their families received a sufficient explanation of the aim and contents of this study before the entry. Patients who provided written informed consent participated in this study. All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation and with Helsinki Declaration of 1975, as revised in 2008.

Data sharing statement: No additional data are available.

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Received: February 23, 2016
Peer-review started: February 23, 2016
First decision: March 7, 2016
Revised: March 19, 2016
Accepted: March 30, 2016
Article in press: March 30, 2016
Published online: June 7, 2016

Abstract

AIM: To elucidate influencing factors of treatment response, then tolvaptan has been approved in Japan for liquid retention.

METHODS: We herein conducted this study to clarify the influencing factors in 40 patients with decompensated liver cirrhosis complicated by liquid retention. Tolvaptan was administered at a dosage of 7.5 mg once a day for patients with conventional diuretic-resistant hepatic edema for 7 d. At the initiation of tolvaptan, the estimated hepatic venous pressure gradient (HVPG) value which was estimated portal vein pressure was measured using hepatic venous catheterization. We analyzed the effects of tolvaptan and influencing factors associated with treatment response.

RESULTS: Subjects comprised patients with a median age of 65 (range, 40-82) years. According to the Child-Pugh classification, class A was 3 patients, class B was 19, and class C was 18. Changes from the baseline in body weight were -1.0 kg (P = 2.04 × 10^{-6}) and -1.3 kg (P = 1.83 × 10^{-5}), respectively. The median HVPG value was 240 (range, 105-580) mmH_2O. HVPG was only significant influencing factor of the weight loss effect. When patients with body weight loss of 2 kg or greater from the baseline was defined as responders, receiver operating characteristic curve analysis showed that the optimal HVPG cutoff value was 190 mmH_2O in predicting treatment response. The response rate was 87.5% (7/8) in patients with HVPG of 190 mmH_2O or less, whereas it was only 12.5% (2/16) in those with HVPG of greater than 190 mmH_2O (P = 7.46 × 10^{-4}). We compared each characteristics factors between responders and non-responders. As a result, HVPG (P = 0.045) and serum hyaluronic acid (P = 0.017) were detected as useful factors.

CONCLUSION: The present study suggests that tolvaptan in the treatment of liquid retention could be more effective for patients with lower portal vein pressure.

Key words: Tolvaptan; V_2 receptor antagonist; Portal vein pressure; Hepatic venous pressure gradient; Decompensated chirrosis

Core tip: To clarify the factors influencing the effect of tolvaptan, a V_2 receptor antagonist, in patients with decompensated liver cirrhosis complicated by liquid retention, we conducted this study. As a result, hepatic venous pressure gradient (HVPG) was the only significant factor that influenced the weight loss effect of tolvaptan. The response rate was 87.5% (7/8) in patients with HVPG of 190 mmH_2O or less, whereas it was only 12.5% (2/16) in those with HVPG of greater than 190 mmH_2O. The present study suggests that tolvaptan in the treatment of liquid retention related to decompensated liver cirrhosis could be more effective for patients with lower portal vein pressure.

Nakagawa A, Atsukawa M, Tsubota A, Kondo C, Okubo T, Arai T, Itokawa N, Narahara Y, Iwakiri K. Usefulness of portal vein pressure for predicting the effects of tolvaptan in cirrhotic patients. *World J Gastroenterol* 2016; 22(21): 5104-5113 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i21/5104.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i21.5104

INTRODUCTION

Liquid retention is a primary complication associated with decompensated liver cirrhosis. Ascites develops at an incidence of approximately 50% within 10 years of the onset of liver cirrhosis[1]. Existence of ascites reduces dietary intakes and deteriorates nutritional statuses, which, in turn, have a negative impact on the quality of life of liver cirrhosis patients[2,3]. Furthermore, the 5-year survival rate after the development of ascites is reportedly 45%[1].

Resting, salt restriction, and therapy with diuretics, such as loop diuretics and anti-aldosterone drugs, have been performed as conventional treatments for ascites related to liver cirrhosis[4,5]. Loop diuretics reduce the reabsorption of sodium and potassium by inhibiting sodium/potassium/chloride cotransporters in the ascending limb of Henle's loop. Anti-aldosterone drugs promote sodium excretion and consequently decrease the excretion of potassium by inhibiting aldosterone receptors. However, the effects of these diuretics are compromised by the progression of liver cirrhosis, leading to electrolyte abnormalities, including hyposodiumemia, a reduction in plasma osmotic pressure, and kidney hypofunction due to a decrease in renal blood flow. The effects of the loop diuretic, furosemide, were previously suggested to be attenuated in patients with liver cirrhosis characterized by a decrease in serum albumin level and reduction in renal blood flow/the glomerular filtration rate[6,7]. If ascites is not improved by these treatments, it is defined as refractory ascites, which is treated with abdominal paracentesis, albumin reinfusion, peritoneal venous shunt (Denver shunt), cell-free and concentrated ascites reinfusion therapy (CART), and transjugular intrahepatic portosystemic shunt (TIPS), but not liver transplantation[4,6,7]. However, there are quite a few patients who are not able to receive these treatments due to complications or conditions that do not meet the indication criteria.

On the other hand, previous studies reported that the V_2 receptor antagonist, tolvaptan, exhibited diuretic effects on heart failure and hyposodiumemia[8-11]. The antidiuretic hormone, vasopressin, enhances water permeability and promotes water reabsorption through V_2 receptors, which exist in the renal collecting ducts. Tolvaptan has been shown to inhibit the vasopressin-related reabsorption of water, thereby increasing water excretion without enhancing the excretion of electrolytes (water-diuretic actions). Since tolvaptan acts on the vascular side around the renal collecting ducts, it differs from the loop diuretic, furosemide. Therefore, its actions are not influenced by a kidney hypofunction-related decrease in the glomerular filtration rate or hypoalbuminemia[12]. Previous studies indicated that tolvaptan prevented conventional diuretic-induced hyposodiumemia in patients with liquid retention[10,13]. Sakaida *et al*[14] conducted a clinical study of tolvaptan for cirrhotic patients with liquid retention and reported increases in the initial 24-h urine volume, even in those with low serum albumin levels. Zhang *et al*[15] indicated that adverse reactions to tolvaptan administration with a daily

dosage of 15 mg included thirst and dry mouth, which were tolerable and safe. Accordingly, tolvaptan for liquid retention in cirrhotic patients who do not respond to conventional diuretics, such as loop diuretics, has been approved in Japan in 2013.

However, not all patients with liquid retention respond to tolvaptan. Furthermore, little is known about the characteristics of patients who respond well to tolvaptan and factors predictive of the therapeutic effect. The present study was conducted to clarify the baseline factors that influence the effect of tolvaptan in cirrhotic patients with conventional diuretic-resistant liquid retention.

MATERIALS AND METHODS

Study design

Forty-seven patients with decompensated liver cirrhosis and liquid retention (pleural effusion, ascites, or lower-limb edema) were recruited for this prospective study in Nippon Medical School Chiba Hokusoh Hospital between September 2013 and August 2015. Patients were eligible for enrollment if they fulfilled the following criteria: (1) patients aged 20 to 85 years; (2) patients diagnosed as liver cirrhosis based on the results of imaging modality (abdominal CT or ultrasonography) or proven by liver biopsy; (3) conventional diuretic-resistant patients in whom liquid retention was not improved with furosemide at a dosage of 20 mg/d or more and/or spironolactone at a dosage of 25 mg/d or more for at least 7 d with salt-restricted diet (5-7 g salinity/day) in-hospital or on an outpatient basis; and (4) patients in whom body weight before breakfast was stable (within the range of ± 1 kg) during the pretreatment observation period. Criteria for exclusion included: (1) uncontrollable hepatocellular carcinoma, such as the Barcelona clinic liver cancer (BCLC) stage D. BCLC stage D is end-stage hepatocellular carcinoma in a patient with disturbed liver function (Child-Pugh C) and/or performance status 3-4, and with an average predicted survival of 3 months; (2) esophageal varices with requiring treatment; (3) existence of portal vein thrombosis based on imaging modality (abdominal CT or ultrasonography); (4) hepatic encephalopathy stage 2 or higher according to The West Haven classification of hepatic encephalopathy including Asterixis[16,17]; (5) type 1 hepatorenal syndrome; and (6) a serum sodium level of 147 mEq/L or higher. All patients and their families received a sufficient explanation of the aim and contents of this study before the entry. Patients who provided written informed consent participated in this study. All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation and with Helsinki Declaration of 1975, as revised in 2008. The protocol was approved by the Ethics Review Board of Nippon Medical School Chiba Hokusoh Hospital (approval No. 526012). All patients and their families received a sufficient explanation of the aim and contents of this study before the entry.

Treatment protocol

Patients were initially instructed to receive salt-restricted diet therapy (5 to 7 g/d) and conventional diuretics for at least 7 d. Tolvaptan (SAMUSKA, Otsuka Pharmaceutical Co., Ltd., Tokyo, Japan) was orally administered at a dosage of 7.5 mg once a day. Water intake was not restricted during the administration of tolvaptan. No albumin preparation was infused, and ascites and pleural effusion were not removed by paracentesis during the first 7 d of tolvaptan treatment. Based on previous clinical studies using tolvaptan, patients with a decrease of 2 kg or greater from the baseline in body weight were regarded as responders[18,19].

Laboratory tests

Body weight and 24-h urine volume were daily measured before the administration of tolvaptan and during at least 7 d of treatment. Body weight was measured at the time of awaking. Clinical symptoms and vital signs (blood pressure, pulse rate, body temperature, and arterial blood oxygen saturation) were closely monitored every day. Biochemical tests (serum sodium, creatinine, urea nitrogen, albumin, and blood ammonia levels) and urinalysis (urinary osmotic pressure) were performed at 1, 3, 5 and 7 d of treatment.

Measurement of portal vein pressure

At the initiation of tolvaptan, the estimated portal vein pressure [*i.e.*, the hepatic venous pressure gradient (HVPG)][20,21] was measured using hepatic venous catheterization to investigate whether or not HVPG influenced the response of tolvaptan, when patients agreed with the optional HVPG measurement study. The right internal jugular vein (or the left internal jugular vein when the right-sided puncture was difficult or failed) was punctured with an 18-gauge needle, and subsequently a 5F sheath (Super Sheath: MEDKIT, Tokyo) was inserted along a guide wire. A 2.9F balloon catheter (Selecon MP catheter 2: TERUMO CLINICAL SUPPLY, Gifu, Japan) was then inserted into the inferior vena cava (IVC) to measure IVC pressure, which was used as a zero adjustment in portal vein pressure measurement. The balloon catheter was further inserted into the right hepatic vein to occlude it with the balloon. Hepatic venography was performed using Iopamidol (Bayer Medicine, Osaka, Japan) to confirm retrograde contrast enhancement involving the portal trunk and the presence of a hepatic vein-hepatic vein shunt and portal thrombosis. Wedged hepatic venous pressure (WHVP) was subsequently measured, and the balloon was removed to determine the free hepatic venous pressure (FHVP). The difference between WHVP and FHVP, which is equal to HVPG, was

Table 1 Demographic and clinical characteristics at baseline

Characteristics	*n* = 40
Age (yr)	65 (40-82)
Gender (M/F)	26/14
Body weight (kg)	61.9 (44.8-88.5)
Liver disease etiology	
Hepatitis B/Hepatitis C/Alcohol/PBC/PSC/NASH	3/15/15/3/1/3
Child-Pugh classification A/B/C	3/19/18
Total bilirubin (mg/dL)	1.0 (0.4-26.2)
Serum albumin (g/dL)	2.6 (1.6-3.7)
Serum creatinine (mg/dL)	0.95 (0.45-6.45)
Serum eGFR (mL/min/1.73 m^2)	60 (8-112)
Serum sodium (mEq/L)	139 (124-146)
Serum hyaluronic acid (ng/mL)	420.7 (122-6984)
BUN (mg/dL)	19 (8.1-81.8)
Urine osmolality (mOsm/L)	414.5 (254-954)
Hepatic venous pressure gradient (mmH_2O)[1]	240 (105-580)
Dose of furosemide (mg/d)	37.0 ± 29.5
Dose of spironolactone (mg/d)	43.4 ± 26.8
Hepatocellular carcinoma (with/without)	12/28
Esophageal varix (with/without)	25/15

[1]Hepatic venous pressure gradient was measured in 24 patients. Categorical variables are given as number. Almost continuous variables are given as median (range). Dose of furosemide and spironolactone are given as mean ± SD). BUN: Blood urea nitrogen; PBC: Pimary biliary cirrhosis; PSC: Primary sclerosing cholangitis; NASH: Nonalcoholic steatohepatitis; eGFR: Estimated glomerular filtration rat.

regarded as the estimated portal vein pressure. Seven days after the start of tolvaptan, HVPG was repeatedly measured using the same procedures to evaluate the influence of tolvaptan on portal vein pressure, when patients agreed with the optional HVPG measurement study.

Statistical analysis

Changes in 24-h urine volumes and body weights after the administration of tolvaptan were evaluated using Wilcoxon's signed rank test. Subjects were divided into two groups based on the medians of baseline values in quantitative variables, and the two groups were compared using the Mann-Whitney *U*-test. Categorical data were analyzed using the Fisher's exact test. The cut-off value of HVPG for the efficacy assessment was calculated using a receiver operating characteristic (ROC) curve. A *P* value of 0.05 was regarded as significant. Excel Statistics 2015 software (SSRI Institute, Tokyo) was used for statistical analyses.

RESULTS

Patients

Among the 47 recruited patients, 7 were excluded from this prospective study: 4 met the exclusion criteria and 3 did not provide informed consent. Therefore, 40 patients were subjected to the clinical study and subsequent analysis. Patient characteristics are shown in Table 1. Patients consisted of 26 males (65.0%) and 14 females (35.0%), with the median age of 65 years (range, 40-82 years). The etiology of liver diseases was hepatitis C for 15 patients, hepatitis B for 3, alcoholic hepatitis for 15, primary biliary cirrhosis for 3, primary sclerosing cholangitis for 1, and non-alcoholic steatohepatitis for 3. According to the Child-Pugh classification, 3 patients were classified into class A, 19 into class B, and 18 into class C. Twelve patients had hepatocellular carcinoma. Twenty-five patients had esophageal varices, but did not require the treatment at the entry. Median serum albumin, sodium and creatinine levels were 2.6 (range, 1.6-3.7) g/dL, 139 (range, 124-146) mEq/L, 0.95 (range, 0.45-6.45) mg/dL, respectively. The median HVPG value and hyaluronic acid value were 240 (range, 105-580) mmH_2O and 420.7 (range, 122-6984), respectively. The median urinary osmotic pressure was 414.5 (range, 254-954) mOsm/L. The daily dosages of furosemide and spironolactone before the administration of tolvaptan were 37.0 ± 29.5 mg and 43.4 ± 26.8 mg, respectively.

Effects of tolvaptan, biochemical tests, and urinalysis

Changes in body weight and 24-h urine volume after the administration of tolvaptan are shown in Figure 1. Median 24-h urine volumes on days 1 and 7 were 1600 mL and 1582 mL, respectively. The median volume increases from the baseline were +492 mL ($P = 6.97 \times 10^{-5}$) and +474 mL ($P = 4.87 \times 10^{-4}$), respectively. The median body weight decreases from the baseline on days 1 and 7 were 1.0 kg ($P = 2.04 \times 10^{-6}$) and 1.3 kg ($P = 1.83 \times 10^{-5}$), respectively.

Patients were divided into two groups based on the median value of each baseline quantitative variable. Changes in body weight loss during 7 d were compared between the two groups (Figure 2A-G). Hyaluronic acid level was a marginally significant factor influencing the weight loss effect. Patients with lower hyaluronic acid level had favorable response to tolvaptan compared with those with higher hyaluronic acid level, though not significant ($P = 0.088$) (Figure 2G).

Association between HVPG and the effect of tolvaptan

Twelve patients rejected measurement of the HVPG. Therefore, the HVPG measurement procedures were performed in 28 patients before the administration of tolvaptan. Of these, 4 were excluded from subsequent analysis due to a hepatic vein-hepatic vein shunt on hepatic venography.

Patients were divided into two groups: those with HVPG of higher than 200 mmH_2O, which is the cutoff value for the diagnosis of portal hypertension and those with HVPG of 200 mmH_2O or lower. The median changes in body weight loss on day 7 were -0.2 kg in the former and -3.05 kg in the latter ($P = 0.012$) (Figure 2H). Using the ROC curve, the cutoff value of 190 mmH_2O (sensitivity: 75.0%, specificity: 93.3%, area under the curve: 0.825) was the most useful in discriminating responders from non-responders (Figure 3). Among patients with HVPG of 190 mmH_2O or lower,

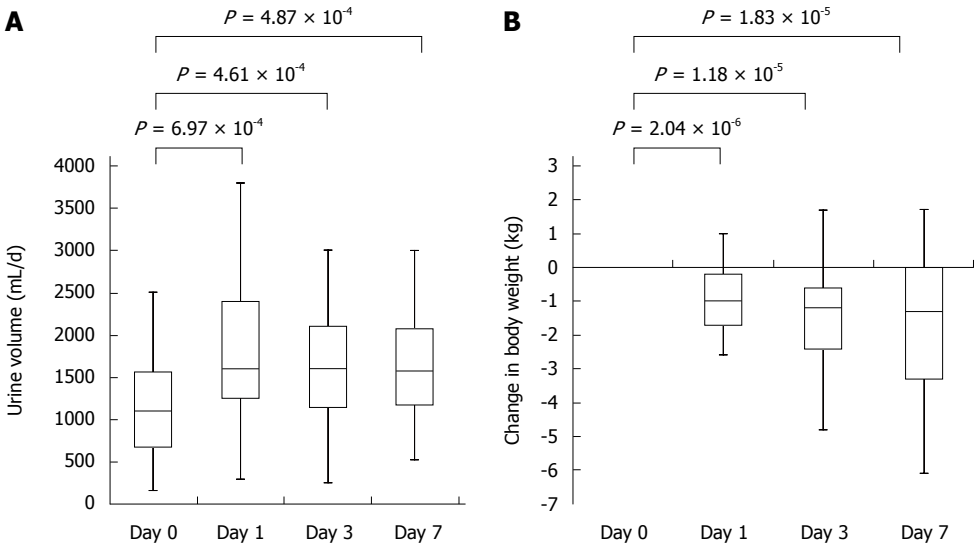
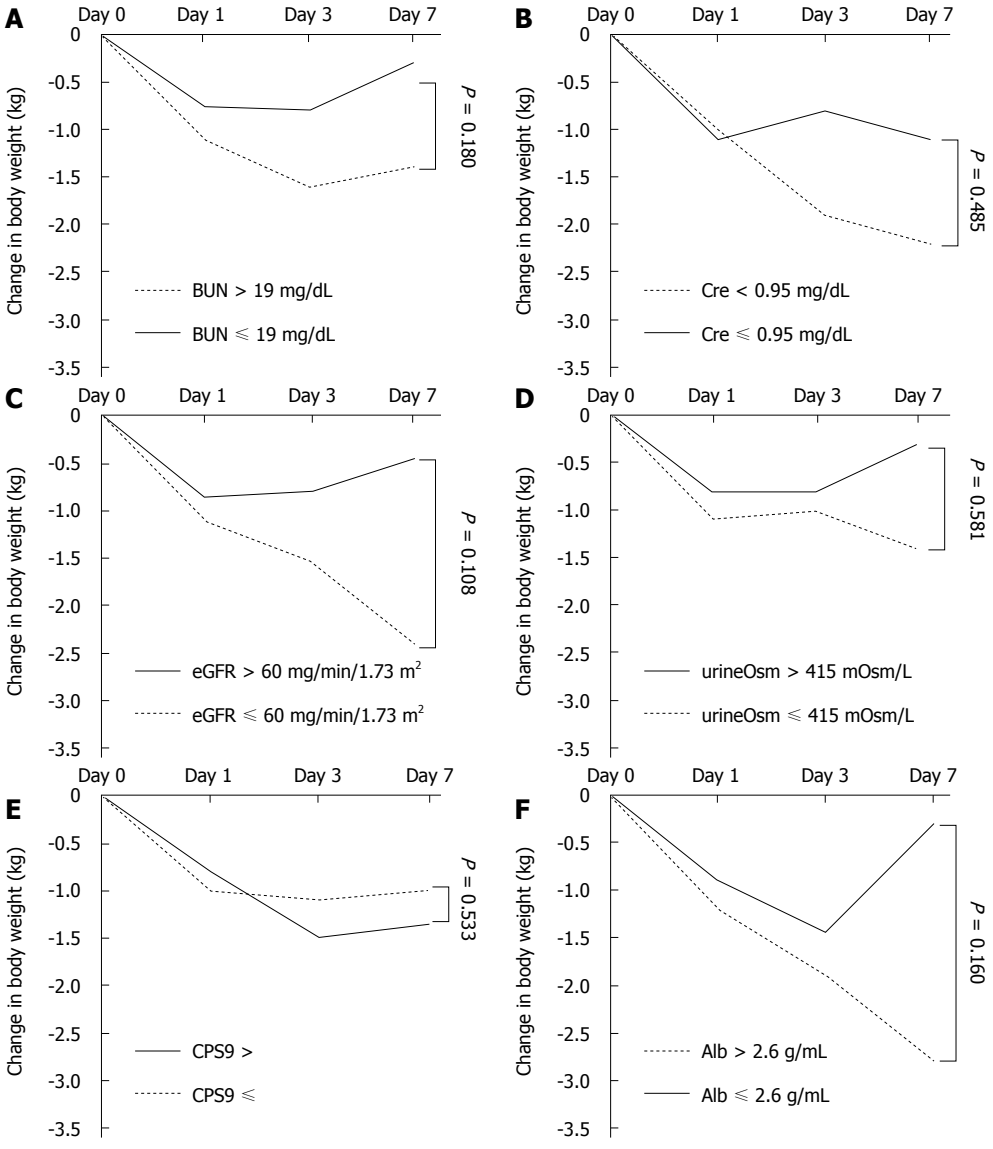


Figure 1 Effects of tolvaptan on liquid retention in all of the patients included in this study. A: Box and whisker plots of daily urine volumes during the first week of tolvaptan administration in all of the patients. Median values were 1108 mL, 1600 mL, 1500 mL and 1582 mL on day 0, 1, 3 and 7, respectively; B: Box and whisker plots of changes in body weight from baseline during the first week of tolvaptan administration in all of the patients. Median changes in body weight were -1 kg, -1.2 kg, and -1.3 kg on day 1, 3 and 7, respectively. Difference between day 0 (= baseline) and day1, 3 and 7 were compared by using Wilcoxon signed rank test.



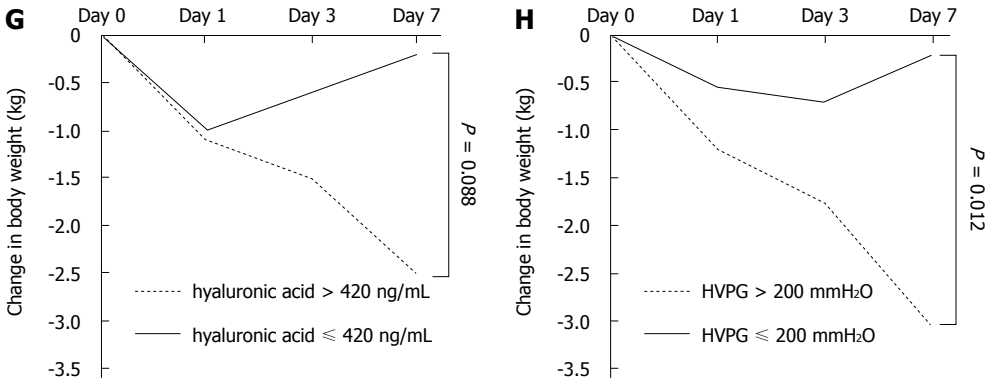


Figure 2 Change in body weight from baseline on each baseline factor during the first week of tolvaptan administration. Data are expressed as median. Patients were divided into two groups using the median value of each baseline variable: (A) serum BUN; (B) serum creatinine; (C) serum eGFR; (D) urine osmolality; (E) Child-Pugh Score (CPS); (F) serum albumin (Alb); (G) serum hyaluronic acid; and (H) Hepatic venous pressure gradient (HVPG).

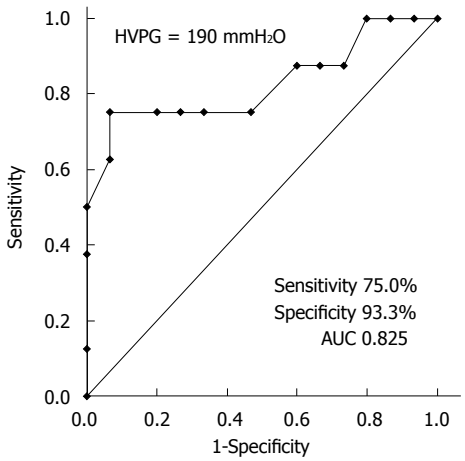


Figure 3 Optimal cutoff value of hepatic venous pressure gradient of the efficacy assessment was determined using ROC curve. The value of 190 mmH2O [sensitivity, 75.0%; specificity, 93.3%; and area under the curve (AUC), 0.825] was the most useful in predicting treatment response, defined as body weight loss of 2 kg or greater from the baseline. HVPG: Hepatic venous pressure gradient.

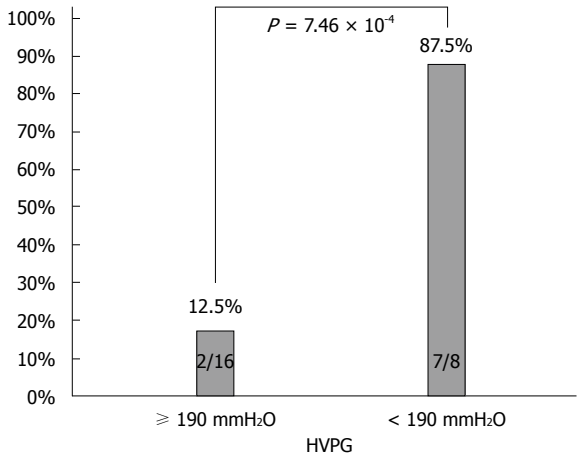


Figure 4 Difference in treatment response rates between patients with low and high hepatic venous pressure gradient. The response rate of 87.5% in the latter with 190 mmH2O or greater was significantly higher than that of 12.5% in the former with less than 190 mmH2O (P = 7.46 × 10^{-4}). HVPG: Hepatic venous pressure gradient.

7 of 8 patients (87.5%) were responders. By contrast, among those with HVPG of higher than 190 mmH2O, only 2 of 16 patients (12.5%) were responders (P = 7.46 × 10^{-4}) (Figure 4).

To examine the influence of tolvaptan on portal vein pressure, changes in HVPG after the administration of tolvaptan were evaluated in 19 patients, in whom the post-treatment HVPG was measured. HVPG values prior to and after the treatment were 213 (range, 105-305) and 210 (range, 150-340) mmH2O, respectively (not significant, P = 0.938, Figure 5A). Even when patients were sub-divided into two groups: those with HVPG of 190 mmH2O and lower (n = 7) and higher than 190 mmH2O (n = 12), no significant changes in HVPG prior to and after the treatment were observed in both subgroups (P = 0.108 and 0.684, respectively; Figure 5B and C).

Differences in background factor according to responses for tolvaptan

Next, based on previous clinical studies using tolvaptan, patients with a body weight decrease of 2 kg or greater from the baseline were regarded as responders. On the other hand, patients with decreases of less than 2 kg or increases from the baseline were regarded as non-responders. We analyzed differences in background factor according to responses for tolvaptan. HVPG (P = 0.045) and serum hyaluronic acid (P = 0.017) were detected as useful factors. All other characteristics factors did not have the significant difference between both groups (Table 2).

Safety

Adverse events were observed in 13 of 40 subjects (32.5%). The most frequent adverse event was pollakiuria, which occurred in six patients (15.0%).

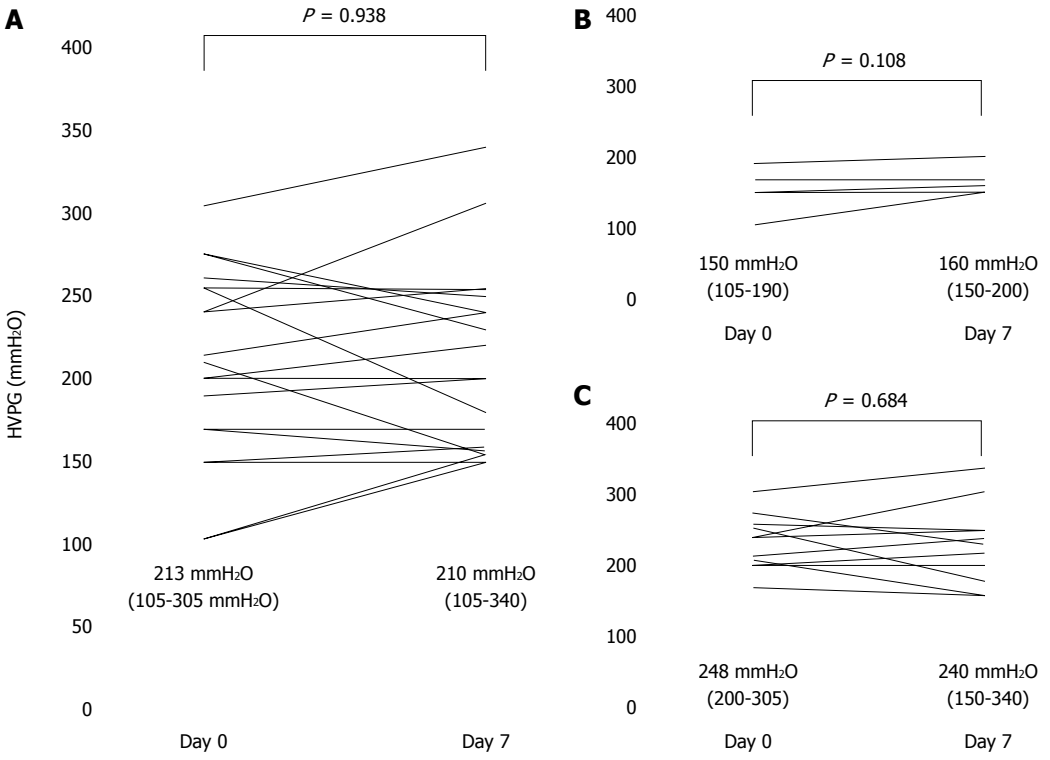


Figure 5 Changes in hepatic venous pressure gradient levels at day 0 and 7. Data are expressed as median (range in parenthesis). A: Overall patients (*n* = 19); B: Patients (*n* = 7) with low HVPG (≤ 190 mmH2O); C: Patients (*n* = 12) with high HVPG (> 190 mmH2O). There was not significant difference in any groups, indicating that tolvaptan had little impact on HVPG. HVPG: Hepatic venous pressure gradient.

Table 2 Comparison of demographic and clinical characteristics at baseline between responders and non-responders

Characteristics	Responder (*n* = 17)	Non-responder (*n* = 23)	*P* value
Age (yr)	66 (45-80)	61 (40-82)	0.8137
Body weight (kg)	62.3 (44.8-88.5)	61.7 (50.3-79.2)	0.7857
Liver disease etiology (Hepatitis B/Hepatitis C/Alcohol/PBC/PSC/NASH)	3/5/5/2/1/1	0/10/10/1/0/2	-
Child-Pugh classification (A-B/C)	9/8	13/10	1.000
Total bilirubin (mg/dL)	0.8 (0.4-11.4)	1.3 (0.5-26.2)	0.332
Serum albumin (g/dL)	2.5 (1.9-3.5)	2.6 (1.6-3.7)	0.733
Serum creatinine (mg/dL)	0.91 (0.62-1.83)	1.1 (0.45-6.45)	0.480
Serum eGFR (mL/min/1.73 m^2)	62 (24-85)	51 (8-112)	0.290
Serum sodium (mEq/L)	137.5 (125-146)	140 (124-144)	0.855
Serum hyaluronic acid (ng/mL)	335 (181-2843)	567.9 (122-6984)	0.017
BUN (mg/dL)	18.1 (8.1-81)	20.9 (10.1-81.8)	0.211
Urine osmolality (mOsm/L)	418 (257-700)	361.5 (254-954)	0.293
Hepatic venous pressure gradient (mmH2O)[1]	170 (105-580)	255 (150-350)	0.045
Hepatocellular carcinoma (with/without)	6/11	6/17	0.729
Esophageal varix (with/without)	10/7	15/8	0.518

[1]Hepatic venous pressure gradient was measured in 24 patients. Categorical variables are given as number. Continuous variables are given as median (range). BUN: Blood urea nitrogen; PBC: Pimary biliary cirrhosis; PSC: Primary sclerosing cholangitis; NASH: Nonalcoholic steatohepatitis.

Thirst was noted in five patients. Malaise was observed in two patients. Serum creatinine levels increased in three patients: one of them discontinued tolvaptan after 5 d of treatment [serum creatinine = 2.34 mg/dL (+1.59 mg/dL from baseline)] and recovered rapidly after the cessation. No other severe adverse events were noted.

DISCUSSION

Tolvaptan was approved as a drug for heart failure in Japan in 2010. Thereafter, its favorable therapeutic effects have been reported[22]. Furthermore, a phase Ⅲ study of tolvaptan for liquid retention was conducted in Japan. Sakaida *et al*[23] reported that body weight decreased by 1.95 kg and 24-h urine volume increased by 633 mL during a 7-d administration period, suggesting the efficacy and safety of tolvaptan in the treatment of liquid retention. In response to the encouraging data, tolvaptan is clinically available in Japan since 2013. In some patients, however, tolvaptan does not improve liquid retention. Little is known about the characteristics of patients who respond well to tolvaptan and the factors influencing the therapeutic effect. Furthermore, the role of tolvaptan in the therapeutic strategy for liquid retention currently remains unclear: whether tolvaptan is used separately from or in combination with conventional diuretics should be determined, and the commencing time of

tolvaptan needs to be clarified.

The present study is the first to show that response to tolvaptan correlated closely with HVPG, which reflects portal vein pressure in cirrhotic patients. Measurement of HVPG makes it possible to estimate the stage of liver fibrosis regardless of disease etiology[24,25], and to assess the severity and prognosis of liver cirrhosis[26,27] and the risk of complications, such as the rupture of esophageal varices, ascites, hepatic encephalopathy, and hepatorenal syndrome[28,29]. Ripoll *et al*[30] reported that decompensated liver cirrhosis was more likely to deteriorate in patients with HVPG of 10.0 mmHg (approximately 136 mmH_2O) or higher. Kumar *et al*[31] found that HVPG of 13.0 mmHg (approximately 177 mmH_2O) or higher was predictive of advanced fibrosis. In the present study, the cut-off value of 190 mmH_2O was the most useful in predicting treatment response, suggesting that tolvaptan exerts its effects on conventional diuretic-resistant patients with lower HVPG. For those with higher HVPG, combination with other treatments, such as TIPS, may be needed to improve tolvaptan-resistant liquid retention. However, HVPG was not decreased even in responders, indicating that tolvaptan has little impact on portal vein pressure. This phenomenon may be attributed to the antagonistic action site of tolvaptan, a vasopressin V_2 receptor, which is in the uriniferous tubules of the kidney alone, and thus does not cause vasoconstriction[12]. In other words, tolvaptan has no anti-vasoconstrictive effect on splanchnic vessels. By contrast, terlipressin, which acts on the vasopressin V_1 receptor, has vasoconstrictive effects on the visceral vessels and consequently reduces portal blood flow[32].

The direct relationship between high portal vein pressure and low responsiveness to tolvaptan is unclear. We examined the correlations between HVPG and various biochemical data in the present study. Although no significant factor could be found, low serum albumin and low eGFR levels might be associated with relatively high HVPG (data not shown). These variables reflect the reserved function of the liver and kidneys. Such patients with impaired liver and kidney functions are likely to have high HVPG, which attenuates the effect of tolvaptan.

The limitations of this study included the small number of patients examined and variations in the etiology of liver diseases. Only the short-term effect of tolvaptan was evaluated, water restriction and water intake were not measured, and drinking-related changes in body fluid volumes were not accurately assessed. However, a response criterion used in the present study (body weight loss of 2 kg or greater) may be appropriate, because the change in body weight after the administration of tolvaptan was reported to correlate with that in ascites volume[33]. Akiyama *et al*[34] administered tolvaptan for 42 d to patients: the initial significant effects lasted during the treatment period, though the mid- to long-term effects of tolvaptan remain controversial. A large-scale clinical trial has not yet been conducted, and it remains unknown whether tolvaptan improves the prognosis of patients with liquid retention.

Since hepatic venous catheterization to measure HVPG is relatively invasive, simple non-invasive tests and biomarkers are required. Previous studies reported that hepatic/splenic stiffness on transient elastography correlated with HVPG[35,36], whereas others indicated that the portal blood flow velocity and intrahepatic passage time measured using contrast-enhanced ultrasonography reflected severe portal hypertension[37]. However, these examinations are not useful in decompensated liver cirrhosis patients with ascites[38]. A recent study reported that the ICG value at 15 min[39] and inflammatory biomarkers, such as IL-1β and VCAM-1, correlated with portal blood pressure[40], though these studies involved only patients with compensated liver cirrhosis. A previous study showed that von Willebrand Factor antigen correlated with HVPG in decompensated liver cirrhosis patients with HVPG of 12 mmHg (approximately 163 mmH_2O) or more[41], though this test is not clinically available. Further studies are needed to develop an easy-to-implement, non-invasive method that sufficiently reflects HVPG even in patients with decompensated liver cirrhosis and predicts the therapeutic effects of tolvaptan.

The present study showed that responders to tolvaptan were likely to have lower HVPG and that tolvaptan had little impact on portal vein pressure. If high portal vein pressure in non-responders is decreased by beta-blocker, splenic artery embolization or TIPS, which could reduce portal vein pressure[42-46], the effect of tolvaptan may be improved. Additive or synergistic effects on liquid retention may be produced by lowering portal vein pressure in combination with these treatments.

In conclusion, the present study suggests that tolvaptan is effective for liquid retention in decompensated liver cirrhosis patients with lower portal vein pressure. By contrast, patients with higher HVPG have the likelihood of treatment failure. In the future, therapeutic strategy needs to be established to treat liquid retention in refractory patients.

ACKNOWLEDGMENTS

We thank Yoshiko Seki (Division of Gastroenterology, Nippon Medical School Chiba Hokusoh Hospital) for their excellent technical support.

COMMENTS

Background

A V_2 receptor antagonist became clinically available in Japan in 2013 for the treatment of liquid retention. On the other hand, factors influencing treatment response have not yet been elucidated. The authors conducted this prospective study to clarify such factors in 40 patients with decompensated liver cirrhosis complicated by liquid retention.

Research frontiers

Changes from the baseline in body weight were -1.0 kg and -1.3 kg on days 1 and 3, respectively. Hepatic venous pressure gradient (HVPG) was only significant factor influencing the weight loss effect of tolvaptan. When patients with body weight loss of 2 kg or greater from the baseline were defined as responders, the response rate was 87.5% (7/8) in patients with HVPG of 190 mmH_2O or less, whereas it was only 12.5% (2/16) in those with HVPG of greater than 190 mmH_2O.

Innovations and breakthroughs

At the initiation of tolvaptan treatment, the HVPG value, which was estimated from portal vein pressure, was measured using hepatic venous catheterization. The authors analyzed factors influencing the effects of tolvaptan including HVPG.

Applications

Since hepatic venous catheterization to measure HVPG is relatively invasive, simple non-invasive tests and biomarkers are required.

Terminology

The present study suggests that tolvaptan in the treatment of liquid retention is more effective for patients with lower portal vein pressure. On the other hand, patients with high portal vein pressure need to be treated by beta-blocker, splenic artery embolization or TIPS, which may reduce portal vein pressure.

Peer-review

The paper is devoted to the analysis of the efficacy of a V_2 antagonist used in heart failure and hyponatemia in cirrhotic patients with severe liquid retention.

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P- Reviewer: Bordas JM **S- Editor**: Ma YJ **L- Editor**: A
E- Editor: Ma S

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Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.3748/wjg.v22.i21.5114

World J Gastroenterol 2016 June 7; 22(21): 5114-5121
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

ORIGINAL ARTICLE

Randomized Controlled Trial

Suppository naproxen reduces incidence and severity of post-endoscopic retrograde cholangiopancreatography pancreatitis: Randomized controlled trial

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Author contributions: Mansour-Ghanaei F and Joukar F designed the study; Mansour-Ghanaei F, Taherzadeh Z and Sokhanvar H collected data; Joukar F contributed to data analysis; Joukar F and Sokhanvar H supervised the procedure; Mansour-Ghanaei F, Joukar F and Hasandokht T drafted the manuscript; all authors read and approved the final manuscript.

Institutional review board statement: The study was reviewed and approved by the ethics committee of the Gastrointestinal and Liver Diseases Research Center of Guilan University of Medical Science.

Clinical trial registration statement: This study is registered at Iranian Registry of Clinical Trials. The registration identification number is IRCT201105301155N14.

Informed consent statement: All study participants, or their legal guardian, provided informed written consent prior to study enrollment.

Conflict-of-interest statement: None declared.

Data sharing statement: No additional data are available.

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Received: June 25, 2015
Peer-review started: June 27, 2015
First decision: October 15, 2015
Revised: October 27, 2015
Accepted: January 17, 2016
Article in press: January 18, 2016
Published online: June 7, 2016

Abstract

AIM: To determine the efficacy of rectally administered naproxen for the prevention of post-endoscopic retrograde cholangiopancreatography (ERCP) pancreatitis (PEP).

METHODS: This double-blind randomized control trial conducted from January 2013 to April 2014 at the Gastrointestinal and Liver Diseases Research Center in Rasht, Iran. A total of 324 patients were selected from candidates for diagnostic or therapeutic ERCP by using the simple sampling method. Patients received a single dose of Naproxen (500 mg; n = 162) or a placebo (n = 162) per rectum immediately before ERCP. The overall incidence of PEP, incidence of mild to severe PEP, serum amylase levels and adverse effects were measured. The primary outcome measure was the development of pancreatitis onset of pain in the upper abdomen and elevation of the serum amylase level to > 3 × the upper normal limit (60-100 IU/L) within 24 h after ERCP. The severity of PEP was classified according to the duration of therapeutic intervention for PEP: mild, 2-3 d; moderate 4-10 d; and severe, > 10 d

and/or necessitated surgical or intensive treatment, or contributed to death.

RESULTS: PEP occurred in 12% (40/324) of participants, and was significantly more frequent in the placebo group compared to the naproxen group ($P < 0.01$). Of the participants, 25.9% (84/324) developed hyperamylasemia within 2 h of procedure completion, among whom only 35 cases belonged to the naproxen group ($P < 0.01$). The incidence of PEP was significantly higher in female sex, in patients receiving pancreatic duct injection, more than 3 times pancreatic duct cannulations, and ERCP duration more than 40 min (Ps < 0.01). There were no statistically significant differences between the groups regarding the procedures or factors that might increase the risk of PEP, sphincterotomy, precut requirement, biliary duct injection and number of pancreatic duct cannulations. In the subgroup of patients with pancreatic duct injection, the rate of pancreatitis in the naproxen group was significantly lower than that in the placebo (6 patients *vs* 23 patients, $P < 0.01$, RRR = 12%, AR = 0.3, 95%CI: 0.2-0.6). Naproxen reduced the PEP in patients with $\geqslant$ 3 pancreatic cannulations ($P < 0.01$, RRR = 25%, AR = 0.1, 95%CI: 0.1-0.4) and an ERCP duration > 40 min ($P < 0.01$, RRR = 20%, AR = 0.9, 95%CI: 0.4-1.2).

CONCLUSION: Single dose of suppository naproxen administered immediately before ERCP reduces the incidence of PEP.

Key words: Naproxen; Nonsteroidal anti-inflammatory drugs; Pancreatic duct injection; Post-endoscopic retrograde cholangiopancreatography; Pancreatitis; Serum amylase

Core tip: Acute pancreatitis is the most common serious complication of endoscopic retrograde cholangiopancreatography (ERCP); prevention of post-ERCP pancreatitis (PEP) has become more challenging. The use of nonsteroidal anti-inflammatory drugs is effective in this condition. This study evaluated the efficacy of rectally administered naproxen for the prevention of PEP in composition with placebo immediately before ERCP.

Mansour-Ghanaei F, Joukar F, Taherzadeh Z, Sokhanvar H, Hasandokht T. Suppository naproxen reduces incidence and severity of post-endoscopic retrograde cholangiopancreatography pancreatitis: Randomized controlled trial. *World J Gastroenterol* 2016; 22(21): 5114-5121 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i21/5114.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i21.5114

INTRODUCTION

Acute pancreatitis is the most common complication of endoscopic retrograde cholangiopancreatography (ERCP), with an incidence rate of 1%-10% that can reach 40% or more, depending on the presence of risk factors[1-5]. Factors predicting post-ERCP pancreatitis (PEP) include young age, female sex, pancreas divisum, sphincter of Oddi dysfunction, previous ERCP-induced pancreatitis, multiple attempts for pancreatic duct cannulation, and pancreatic duct injection[6,7]. Although most cases of PEP are clinically mild or moderate in severity, 10% present severe manifestations[8,9].

ERCP complications may be minimized by reducing pancreatic secretion, interrupting inflammatory cascades, relaxing the sphincter of Oddi, and preventing intra-acinar trypsinogen activation infection[10,11]. Several pharmacologic agents, including octreotide[12], diclofenac[13,14], and recombinant interleukin-10[15,16], have been investigated to reduce the incidence and severity of PEP. Additionally, the protease inhibitors gabexate mesylate[17], and somatostatin[18,19] had been shown are effective in preventing PEP, particularly when administered as an intra-venous infusion[20,21].

Evidence suggests that the patient's inflammatory response to pancreatic duct imaging and instrumentation contribute to the development of PEP[7,22-27]. As phospholipase A_2 may play a vital role in the initial inflammatory cascade of acute pancreatitis[24], identifying pharmacologic agents that inhibit or disturb this cascade may prevent or limit the pancreatitis and its consequences. In some randomized controlled trials, various Oral and suppository of nonsteroidal anti-inflammatory drugs (NSAIDs) have shown promising prophylactic activity with regard to PEP[13,14,28-30]. However, different result were seen about the effectiveness of the intraduodenal indomethacin[30,31]. NSAIDs are easily administered, inexpensive, and relatively safe when given as a single dose, making them an attractive treatment option. Despite these benefits of NSAIDs and findings of the recent meta-analysis[32,33] indicated that rectal diclofenac or indomethacin reduce the incidence and severity of PEP, results of the several studies appear to contradict these conclusions[10,34-36]. Tilak Shah *et al*[35] mentioned several questions in this area; comparison between various NSAID, higher dose of drug and different rout of administration. Additionally, there are some study[37,38] reported NSAIDs can cause acute pancreatitis with the highest risk for diclofenac (OR = 5.0, 95%CI: 4.2-5.9) and the lowest for naproxen (OR = 1.1, 95%CI: 0.7-1.7)[39]. Therefore, we conducted a double-blind, randomized, and controlled clinical trial to evaluate the prophylactic effect of a naproxen suppository for the prevention of PEP.

MATERIALS AND METHODS

Study population

Participants were serially enrolled as they were seen for diagnostic or therapeutic ERCP at the gastroenterology ward of Razi Hospital, a referral center in Rasht, Iran between January 2013 and April 2014. Patients over 18 years of age who were scheduled to undergo ERCP and willing to provide written informed consent for study participation were included. Patients who had acute or active pancreatitis, a history of chronic pancreatitis and/or previous endoscopic sphincterotomy, active peptic ulcer disease, rectal disease, aspirin-induced asthma, use of NSAIDs during the preceding two weeks, hypersensitivity to NSAIDs, renal dysfunction, or were pregnant or breastfeeding, were ineligible for the study.

Study design

The protocol for this randomized, controlled clinical trial (IRCT201105301155N14) was approved by the ethics committee of the Gastrointestinal and Liver Diseases Research Center of Guilan University of Medical Science, and written informed consent (per the Helsinki declaration) was obtained from each participant. Eligible participants (n = 324) were selected from candidates for diagnostic or therapeutic ERCP by using the simple sampling method. The sample size was based on the frequency of pancreatitis in the placebo group (P_1 = 0.1)[28] compared to the study drug group (P_2 = 0.04) with α = 0.05 and β = 20%. Selected patients were randomly assigned using permuted-block randomization to receive either a naproxen (500 mg; Behvazan Pharmacy Co., Tehran, Iran) (n = 162) or placebo (n = 162) suppository immediately before ERCP. Concealed envelopes with naproxen or placebo, which appeared identical, were dispensed in sequence. Study participants, ERCP physicians, and nurses who administered treatment were unaware of the nature of the drugs. The group assignment was only known by the programmer of the database used during the study.

ERCP was performed by using a standard therapeutic duodenoscope (Olympus CO.) with the patient under local anesthesia with 2% lidocaine and after premedication by intravenous administration of 0.05 mg/kg of Midazolam or in cases with contraindication, intravenous administration 1 mg/kg pethidine. Blood pressure, heart rate, and oxygen saturation were monitored with automated devices. Contrast medium (Meglumine Compound 76%) was injected manually, under fluoroscopic guidance. ERCPs were carried out by 3 experienced endoscopists, with a mean number of sphincterotomy procedures performed of about 5 to 7 per week.

Outcome and data measurements

The primary outcome measure was the development of pancreatitis, defined according to the guideline of Cotton *et al*[7] as onset of pain in the upper abdomen and elevation of the serum amylase level to > 3 × the upper normal limit (60-100 IU/L) within 24 h after ERCP. The severity of PEP was classified according to the duration of therapeutic intervention for PEP: mild, 2-3 d; moderate 4-10 d; and severe, > 10 d and/or necessitated surgical or intensive treatment, or contributed to death[7].

Serum amylase was measured before, 2 h after, and any time the patients complained of pain within 24 h after ERCP; otherwise, it was routinely measured 24 h after ERCP. After 2 h, patients with normal serum amylase or no history of abdominal pain, nausea and vomiting were permitted to resume oral intake. All patients with prolonged pancreatitis symptoms (> 48 h) were assessed for complications of pancreatitis (abscess, pseudocyst, or fluid collection) by CT.

Demographic characteristics, risk factors, ERCP procedural elements, and follow-up data were collected at the time of the procedure and 24 h after ERCP by a trained physician who was unaware of study-group assignments. ERCP duration, the number of biliary and pancreatic cannulations, findings of the biliary and/or pancreatic duct, and interventions such as sphincterotomy, papillary balloon dilation, and stenting were recorded.

Table 1 Patient characteristics *n* (%)

Characteristic	Naproxen (n = 162)	Placebo (n = 162)
Age, yr	46.3 ± 8.3	44.7 ± 9.7
Female	78 (48.1)	73 (45.1)
Pancreatitis severity		
Mild	8 (20.0)[b]	18 (45.0)
Moderate	4 (10.0)[b]	10 (25.0)
Severe	0 (0)[b]	0 (0)

[b]P < 0.01 *vs* Placebo. Data are presented as mean ± SD or n (%).

Statistical analysis

A two-tailed χ^2 test was used to analyze the difference in the proportion of patients with PEP in the naproxen and placebo groups. Data are expressed as odds ratio (OR) with 95% confidence interval (CI). Additional exploratory subgroup analyses were performed according to age, sex, and procedure, and are reported as relative risk (RR), absolute risk (AR) and relative risk reduction (RRR). All comparisons were carried out on a two-tailed basis. Statistical analysis was carried out with the SPSS (version 16) and P < 0.05 was considered statistically significant. Ninety-five percent significant intervals (CI) for the proportions were calculated.

RESULTS

There were 78 (48.1%) women in the naproxen group and 73 (45.1) women in the control group. The mean age ± SD of the patients in the intervention group was

Table 2 Incidence of post-endoscopic retrograde cholangio-pancreatography pancreatitis

Variable	Naproxen[1]	Placebo[2]
Sex		
Female	9/12[b]	19/28
Male	3/12	9/28
Age (yr)		
< 40	5/12	10/28
> 40	7/12	18/28
Sphincterotomy		
Yes	8/129	23/119
No	4/33	5/43
Precut required	2/31	2/31
Pancreatic duct injection		
Yes	6/75[b]	23/84
No	6/82	5/83
Pancreatic duct cannulations		
⩾ 3	2/6[b]	10/23
⩽ 2	3/6	14/23
ERCP duration (min)		
> 40	4/12[b]	12/28
< 40	8/12	16/28
Biliary duct injection		
Yes	9/134	24/135
No	3/23	4/25

[1]Pancreatitis/naproxen (12/162, 7.4%); [2]Pancreatitis/placebo (28/162, 17%). [b]$P < 0.01$ *vs* Placebo.

46.3 ± 8.3, and in the control group it was 44.7 ± 9.7. The characteristics of trial participants are presented in Table 1. PEP occurred in 12% (40/324) of participants, and was significantly more frequent in the placebo group (28/162, 17%) compared to the naproxen group (12/162, 7.4%) ($P < 0.01$). Of the participants, 25.9% (84/324) developed hyperamylasemia within 2 h of procedure completion, among whom only 35 cases belonged to the naproxen group ($P < 0.01$).

Analyses in different group indicated that the incidence of PEP was significantly higher in patients receiving pancreatic duct injection, cases with 3 times or more pancreatic duct cannulations, ERCP duration > 40 min and female sex (*P*s < 0.01) (Table 2). Logistic regression analysis of possible risk factors for PEP indicated that pancreatic duct injection, duration of ERCP, female sex and age were significant risk factors (*P*s < 0.05) (Table 3). There were no statistically significant differences between the groups regarding the procedures or factors that might increase the risk of PEP, including, sphincterotomy, precut requirement and biliary duct injection.

In the subgroup of patients with pancreatic duct injection, the rate of pancreatitis in the naproxen group was significantly lower than that in the placebo group (6 patients *vs* 23 patients; $P < 0.01$, RRR = 12%, AR = 0.3, 95%CI: 0.2-0.6). Naproxen reduced the PEP in patients with ⩾ 3 pancreatic cannulations ($P < 0.01$, RRR = 25%, AR = 0.1, 95%CI: 0.1-0.4) and an ERCP duration > 40 min ($P < 0.01$, RRR = 20%, AR = 0.9, 95%CI: 0.4-1.2).

The most common final diagnosis after ERCP was choledocholithiasis, followed by several cases of sphincter of Oddi dysfunction, common bile duct tumors, and choledochal cysts (Figure 1). We did use pancreatic duct stenting in the nobody of study subjects. All patients were discharged in good health and there were no reported side effects.

DISCUSSION

A systematic review of five clinical trials[39], as well as two subsequent meta-analyses[40,41], indicate that administration of NSAIDs significantly decreases the incidence of PEP. Our findings show that a single dose of suppository naproxen given immediately before ERCP significantly reduces the overall incidence and severity of PEP. Elmunzer *et al*[42] in a multicenter, randomized, placebo-controlled, double-blind clinical trial showed post-ERCP pancreatitis developed in 16.9% of placebo group *vs* 9.2% in the indomethacin group, as well as, indomethacin decrease the incidence of moderate to severe PEP. Andrade-Dávila *et al*[43] conducted a controlled clinical trial where patients at least one major and/or two minor risk factors for developing post-ERCP pancreatitis. They suggested rectal indomethacin reduced the incidence of post-ERCP pancreatitis among patients at high risk of developing this complication. In our randomized controlled trial, the number of patients who would need to be treated to prevent one episode of pancreatitis was 10. Sethi *et al*[44] in a meta analysis concluded rectal NSAIDs can decrease PEP with 11 patients needed to treatment. However, another meta-analysis showed the number needed to treat was 17[30]. On the other hand, recent controlled clinical trial a number needed to treat of 6.5 patients were calculated to prevent an episode of post-ERCP pancreatitis[43].

The occurrence of PEP varies according to patient characteristics and the type of intervention performed. We found that pancreatic duct injection, duration of ERCP (> 40 min), female sex and age (< 40 year) were significant risk factors for developing PEP, consistent with other studies[9,14,25,42]. European Society of Gastrointestinal Endoscopy Guideline presented cannulation attempts duration > 10 min and younger age increase the incidence of PEP[29]. Similarly, Sotoudehmanesh *et al*[14] demonstrated the protective effect of indomethacin for PEP in the patients with pancreatic duct injection. Furthermore, Elmunzer *et al*[42] showed that indomethacin significantly reduced the risk of moderate to severe PEP from 16.1% to 9.7% in patients with pancreatic injections. In our study, the risk of PEP was not associated with undergoing sphincterotomy, having sphincter of Oddi dysfunction. These findings are in contrast to those of Murray *et al*[13] who found that diclofenac was protective in patients who had sphincterotomy and those without sphincter of Oddi hypertension. Furthermore, recent guideline updated in 2014[29], female gender presented

Table 3 Risk factors for post-endoscopic retrograde cholangiopancreatography pancreatitis

Variable	Pancreatitis, *n*		OR	RR (95%CI)	RRR (%)	AR (%)
	Yes	No				
Group						
Naproxen	12	150	0.4[b]	0.42 (0.22-0.81)	58	-138
Placebo	28	134				
Sex						
Female	28	123	3[b]	2.67 (1.40- 5.07)	167	62.54
Male	12	161				
Age (yr)						
< 40	15	63	2.4[b]	2.2 (1.22-3.96)	120	54.54
> 40	25	261				
Sphincterotomy						
Yes	31	217	1	1.05 (0.52-2.11)	5	4.76
No	9	67				
Pancreatic duct injection						
Yes	29	130	2.1[b]	1.88 (1.06-3.32)	88	46.81
No	16	149				
Pancreatic duct cannulations						
⩾ 3	12	37	2.1	1.87 (0.93-3.74)	87	46.52
⩽ 2	14	93				
ERCP duration (min)						
> 40	16	54	2.6[b]	2.30 (1.29-4.09)	130	56.52
< 40	24	218				
Biliary duct injection						
Yes	33	236	1.7	1.66 (0.76-3.63)	66	39.75
No	7	88				

[b]$P < 0.01$ *vs* Placebo. OR: Odds ratio; RR: Relative risk; RRR: Relative risk reduction; AR: Absolute risk.

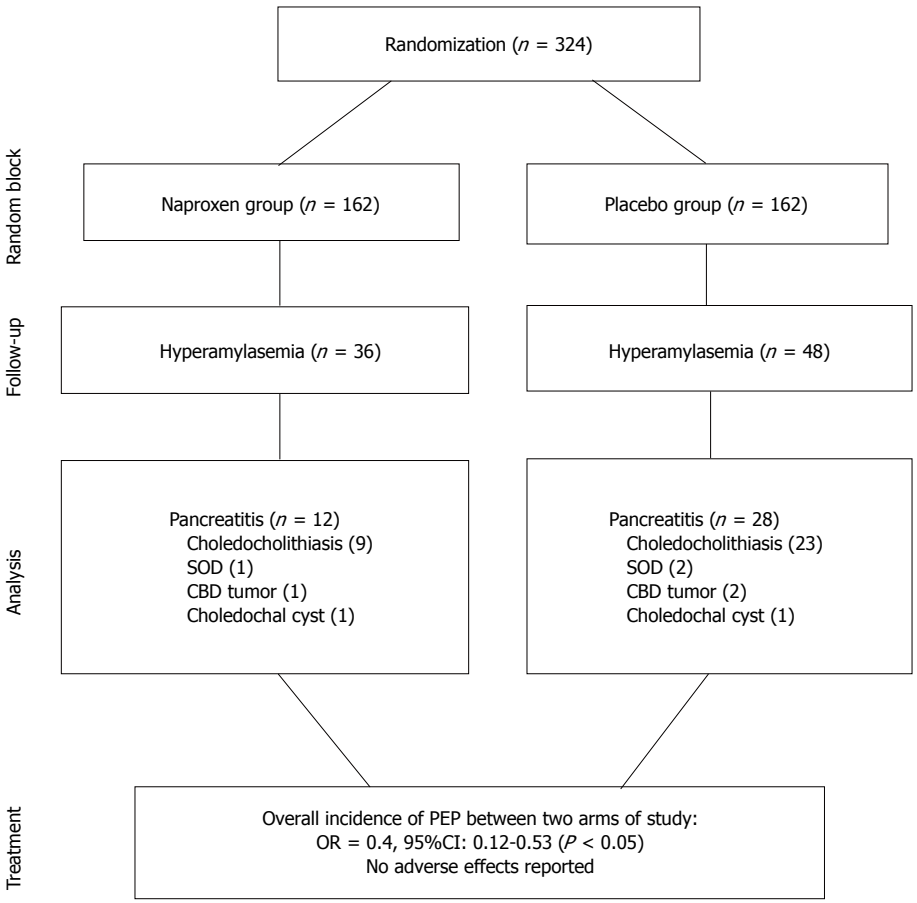

Figure 1 Participant selection. CBD: Common bile duct; PEP: Post-endoscopic retrograde cholangiopancreatography pancreatitis; SOD: Sphincter of Oddi dysfunction.

as a risk factor for PEP. In parallel our study, in recent controlled clinical trial, Suspected sphincter dysfunction oddi were not risk factor for PEP[43]. Till now, several meta-analyses[3,30,39-41,45] conclude that NSAIDs used in the different routes of administration decrease the incidence of pancreatitis and severity of pancreatitis. Although, the results of our study are relevant because the drug was rectally administrated immediately before procedure, the main difference between our study and those previously reported was the use of naproxen instead of indomethacin or diclofenac. Hence, to support the conclusion, a high-quality multicenter randomized clinical trial is required to better describe the effectiveness of naproxen as a NSAID.

In conclusion, a single-dose prophylactic naproxen suppository significantly decreases the occurrence and severity of PEP, particularly in those with pancreatic duct injections, multiple pancreatic duct cannulation attempts, those younger than 40 years of age, and requiring a procedure lasting more than 40 min. Moreover, this treatment produced no adverse events, consistent with previous works[10,28,40], and should therefore be administered immediately before ERCP procedures.

ACKNOWLEDGMENTS

We would like to thank all the Gastrointestinal and Liver Diseases Research Center personnel.

COMMENTS

Background

Acute pancreatitis is the most feared complication of endoscopic retrograde cholangiopancreatography (ERCP) because it has the greatest potential for causing prolonged hospitalization, major morbidity, and occasionally death.

Research frontiers

Acute pancreatitis is the most common complication of ERCP; prevention of post-ERCP pancreatitis (PEP) has become more challenging.

Innovations and breakthroughs

Prevention of PEP has become more challenging. The use of nonsteroidal anti-inflammatory drugs (NSAIDS) is effective in this condition but selection the best effective drug is required more examination of it. This study is based on this real.

Applications

This study evaluated the efficacy of rectally administered Naproxen for the prevention of PEP in patients received a single dose of naproxen or a placebo immediately before ERCP.

Peer-review

This study provides useful information for prevention of PEP. The authors show that a single dose of suppository naproxen administered immediately before ERCP reduces the incidence and severity of PEP.

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P- Reviewer: Gonzalez-Ojeda A, Li SD, Lorenzo-Zuniga V
S- Editor: Gong ZM **L- Editor**: A **E- Editor**: Zhang DN

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DOI: 10.3748/wjg.v22.i21.5122

World J Gastroenterol 2016 June 7; 22(21): 5122-5131
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

META-ANALYSIS

Contemporary meta-analysis of short-term probiotic consumption on gastrointestinal transit

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Supported by Danisco Sweeteners.

Conflict-of-interest statement: Miller LE is a consultant to Bio-K Plus, DuPont, Fonterra, Natren, and UAS Laboratories. Ouwehand AC is an employee of DuPont Nutrition and Health. Zimmermann AC denies any conflict of interest.

Data sharing statement: No additional data are available.

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Received: December 4, 2015
Peer-review started: December 7, 2015
First decision: January 28, 2016
Revised: February 9, 2016
Accepted: March 1, 2016
Article in press: March 2, 2016
Published online: June 7, 2016

Abstract

AIM: To determine the efficacy of probiotic supplementation on intestinal transit time (ITT) in adults and to identify factors that influence these outcomes.

METHODS: We conducted a systematic review of randomized controlled trials of probiotic supplementation that measured ITT in adults. Study quality was assessed using the Jadad scale. A random effects meta-analysis was performed with standardized mean difference (SMD) of ITT between probiotic and control groups as the primary outcome. Meta-regression and subgroup analyses examined the impact of moderator variables on SMD of ITT.

RESULTS: A total of 15 clinical trials with 17 treatment effects representing 675 subjects were included in this analysis. Probiotic supplementation was moderately efficacious in decreasing ITT compared to control, with an SMD of 0.38 (95%CI: 0.23-0.53, $P < 0.001$). Subgroup analyses demonstrated statistically greater reductions in ITT with probiotics in subjects with *vs* without constipation (SMD: 0.57 *vs* 0.22, $P < 0.01$) and in studies with high *vs* low study quality (SMD: 0.45 *vs* 0.00, $P = 0.01$). Constipation ($R^2 = 38\%$, $P < 0.01$), higher study quality ($R^2 = 31\%$, $P = 0.01$), older age ($R^2 = 27\%$, $P = 0.02$), higher percentage of female subjects ($R^2 = 26\%$, $P = 0.02$), and fewer probiotic strains ($R^2 = 20\%$, $P < 0.05$) were predictive of decreased ITT with probiotics in meta-regression. Medium to large treatment effects were identified with *B. lactis* HN019 (SMD: 0.67, $P < 0.001$) and *B. lactis* DN-173 010 (SMD: 0.54, $P < 0.01$) while other probiotic strains yielded negligible reductions in ITT relative to control.

CONCLUSION: Probiotic supplementation is moderately efficacious for reducing ITT in adults. Probiotics were most efficacious in constipated subjects, when evaluated in high-quality studies, and with certain probiotic strains.

Key words: Constipation; Gastrointestinal; Intestinal transit time; Meta-analysis; Probiotics

Core tip: We performed a contemporary systematic review and meta-analysis of randomized controlled trials to determine the effects of short-term probiotic supplementation on transit time in adults. Probiotic supplementation is moderately efficacious for reducing intestinal transit time in adults. Probiotics were most efficacious in constipated subjects, when evaluated in high-quality studies, and with certain probiotic strains.

Miller LE, Zimmermann AK, Ouwehand AC. Contemporary meta-analysis of short-term probiotic consumption on gastrointestinal transit. *World J Gastroenterol* 2016; 22(21): 5122-5131 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i21/5122.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i21.5122

INTRODUCTION

The human colonic microbiota is a complex ecosystem involved in maintenance of health and physiological functions of the host. Disturbances within the microbiota may result in gastrointestinal disorders such as constipation, irritable bowel syndrome, or periodic bouts of irregularity. Functional gastrointestinal disorders are a highly prevalent group of persistent and recurring conditions with a prevalence of 69% in the general population[1]. Slow intestinal transit is a common manifestation of functional gastrointestinal disorders affecting the bowel[2] and may also occasionally affect otherwise healthy individuals. Although the benefits of reducing intestinal transit time (ITT) in patients with constipation are obvious, reductions in ITT are also considered a beneficial physiological effect in the non-diseased general population[3]. Over-the-counter and prescription medications intended to normalize intestinal transit are widely utilized although no known treatment is considered efficacious, safe, and cost effective[4]. Probiotics are live micro-organisms that confer a health benefit on the host when administered in adequate dosages[5] and have been extensively studied for enhancement of gastrointestinal health[6,7]. Previously, we performed the first systematic review and meta-analysis on the efficacy of probiotic supplementation on ITT in adults[8]. The purpose of this study was to update these findings with data from randomized controlled trials (RCTs) published over the 3-year period since our last review.

MATERIALS AND METHODS

Literature search

This study was performed according to the Preferred Reporting Items for Systematic Reviews and Meta-analyses (PRISMA)[9]. We searched MEDLINE and EMBASE for RCTs of probiotic supplementation that reported ITT in adults by using a combination of relevant keywords. The details of the MEDLINE search strategy are listed in Table 1. The syntax for EMBASE was similar, but adapted as necessary. Additionally, manual searches were conducted using the Directory of Open Access Journals, Google Scholar, and the reference lists of included papers and other relevant meta-analyses. No date restrictions were applied to the searches. The final search was conducted in October 2015.

Table 1 MEDLINE search strategy

Therapeutic search terms
Probiotic
Synbiotic
Lactobacill
Bifidobacteri
Yogurt (yoghurt)
Fermented milk
Main outcome search terms
Gastrointestinal
Transit
Gut
Motility
Colonic
Constipation
Irritable bowel
Combination terms
or/1-6
or/7-13
and/14-15

Study selection

Two researchers independently selected studies for inclusion in the review. Disagreements were resolved by consensus. Titles and abstracts were initially screened to exclude manuscripts published in non-English journals. Next, review articles, commentaries, letters, and case reports were excluded. Lastly, we excluded studies of subjects where ITT reduction was undesirable or uninterpretable (*e.g.*, diarrhea or mixed IBS subtypes). Full-text of the remaining manuscripts was then retrieved and reviewed. Publications that failed to report ITT or that described non-randomized, non-controlled, or otherwise irrelevant studies were also excluded.

Data extraction

Data were extracted from eligible peer-reviewed articles by one author and then verified by a second author. Data extraction discrepancies between the two researchers were resolved by consensus. The following variables were recorded in a pre-designed database: general manuscript information (author, institution name and location, journal, year, volume, page numbers), study design characteristics (study quality, study design, sample size, method of ITT assessment,

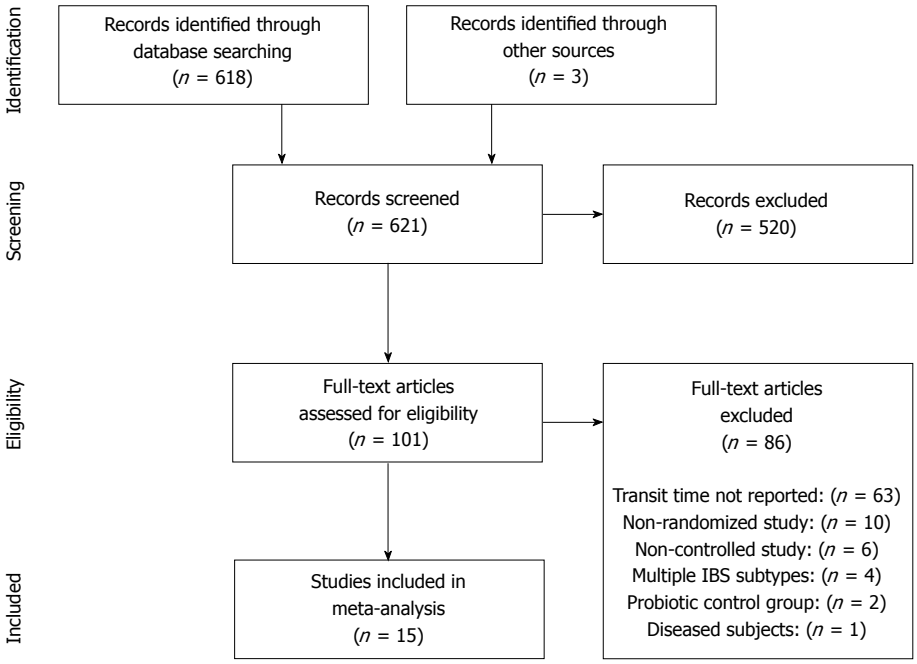

Figure 1 PRISMA flow diagram.

probiotic strain, daily dosage, product delivery method, and treatment duration), subject characteristics (age, gender, body mass index, and condition), and ITT summary statistics necessary for meta-analysis.

Quality assessment

The Jadad scale was used to assess RCT study quality[10]. Studies were scored according to the presence of three key methodological features: randomization, blinding and subject accountability. Randomization was scored from 0 to 2, blinding was scored from 0 to 2, and subject accountability was scored 0 or 1. RCTs with a score of 3 to 5 were classified as high quality; studies with a score of 0 to 2 were classified as low quality.

Statistical analysis

A random effects meta-analysis model was selected *a priori* based on the assumption that treatment effects were heterogeneous given the differences in probiotic strain, study design characteristics, and subject characteristics among studies. The standardized mean difference (SMD) and 95% confidence interval (CI) were the statistics of interest to describe treatment effects since different measures of ITT (*e.g.*, whole gut, colonic, oro-cecal, *etc*.) were utilized in the included studies. The SMD is calculated as the mean difference in ITT between probiotic and control groups divided by the pooled standard deviation in ITT. SMD values of 0.2, 0.5, and 0.8 are defined as small, medium, and large, respectively[11]. Positive SMDs imply that probiotics were more effective in reducing ITT *vs* control while negative SMDs imply a greater treatment effect with control *vs* probiotics. A forest plot was used to illustrate the individual study findings and the random effects meta-analysis results. Heterogeneity of effects across studies was estimated with the I^2 statistic where values of $\leqslant$ 25%, 50%, and $\geqslant$ 75% represent low, moderate, and high inconsistency, respectively[12]. In addition, a one study removed meta-analysis was performed to assess the influence of individual studies on the meta-analysis findings. Publication bias was visually assessed with a funnel plot and quantitatively assessed using Egger's test[13]. Meta-regression and subgroup analyses were performed to explore sources of heterogeneity. All analyses were performed using Comprehensive Meta-analysis (version 2.2, Biostat, Englewood NJ). The statistical methods of this study were reviewed by Clinton Hagen, MS (Mayo Clinic, Rochester, MN).

RESULTS

Study selection

Our initial database search retrieved 618 titles and abstracts; hand searching relevant bibliographies identified 3 additional records. After screening records for inclusion criteria, 101 full text articles were reviewed for eligibility. Ultimately, 15 RCTs with 17 treatment effects representing 675 unique subjects were included in the final analysis[14-28]. A flow chart of study identification and selection is shown in Figure 1.

Study characteristics

Sample sizes ranged from 10 to 36 per treatment arm for parallel groups designs (9 studies) and from 12 to 83 for cross-over designs (6 studies). Thirteen RCTs contributed one treatment effect each and two RCTs contributed two effects each; the study of Rosenfeldt

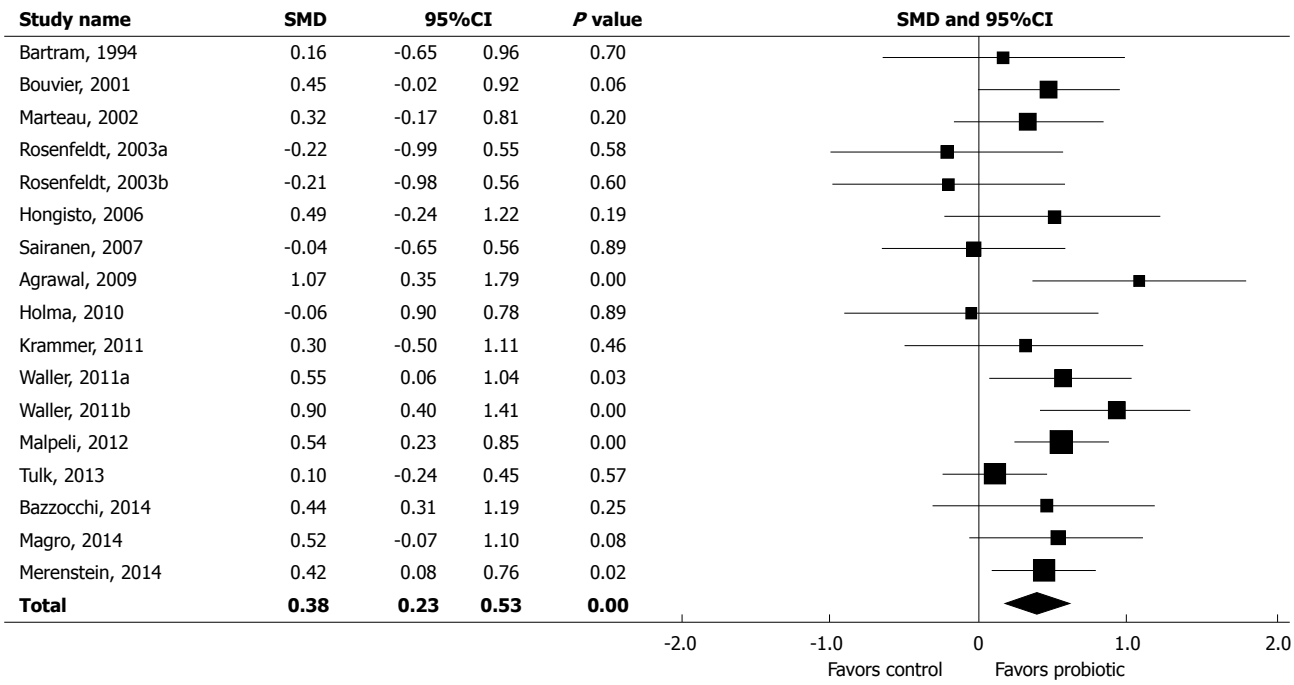

Figure 2 Forest plot of standardized mean difference in intestinal transit time across studies. Random effects model. I^2 = 20%, P = 0.22. SMD: Standardized mean difference.

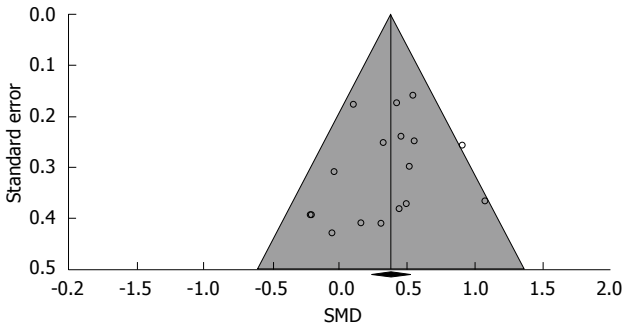

Figure 3 Funnel plot of standardized mean difference in intestinal transit time across studies. Egger's P value = 0.44 for publication bias. SMD: Standardized mean difference.

and colleagues[21] assessed two different probiotic formulations and the study of Waller and colleagues[23] assessed two different dosages of the same probiotic strain. Daily probiotic dosages varied considerably among studies, ranging from 5×10^8 to 9.8×10^{10} colony forming units (CFU) per day (median 1.6×10^{10} CFU per day). Probiotic treatment periods ranged from 10 to 28 d (median 18 d). Intestinal transit time was measured using radiopaque markers in 13 studies and with carmine red dye in 2 studies. The most commonly tested product format was yogurt or other forms of fermented milk. Six (40%) studies included other components in the active product known to influence ITT such as lactulose, psyllium, inulin, polydextrose, maltodextrose, and oligofructose (Table 2).

Subject characteristics

Nine treatment effects were calculated for subjects with constipation or IBS-C while 8 effects were based on healthy subjects. Subjects were predominantly female, mean age ranged from 23 to 50 years, and mean body mass index ranged from 19 to 32 kg/m^2 (Table 3).

Study quality assessment

Overall, the quality of RCT reporting was medium with a median Jadad score of 3 (range: 1-5). Twelve of 17 treatment effects were based on high quality (Jadad score 3-5) trials. The method of randomization was inadequately described in most studies. Descriptions of blinding were adequate overall. Subject accountability in RCTs was sufficiently detailed in 11 of 17 cases (Table 4).

Main results

In relation to controls, probiotic supplementation statistically decreased ITT, with an SMD of 0.38 (95%CI: 0.23-0.53, $P < 0.001$) (Figure 2). Only 5 of 17 treatment effects statistically favored probiotic supplementation. There was low heterogeneity among studies (I^2 = 20%, P = 0.22) with no evidence of publication bias (Egger's regression test: P = 0.44) (Figure 3). A one study removed sensitivity analysis was performed to determine the influence of individual studies on main outcomes. Overall, no single study significantly influenced the observed SMD of ITT with probiotics *vs* control. SMDs ranged from 0.35 to 0.42 (all $P < 0.001$) following removal of each study one at a time from the meta-analysis (Figure 4).

Additional analyses

Subgroup analyses (SA) (Table 5) and meta-regression

Table 2 Study characteristics

Study	Country	Study design	*n* (active: control)	Transit time outcome, method	Probiotic strain	Daily dosage (10^9 CFU)	Delivery method	Treatment duration (d)
Agrawal *et al*[14], 2009	United Kingdom	Parallel groups	17:17	CTT, radiopaque markers	*B. lactis* DN-173 010	25	Active: Yogurt + probiotic Control: Nonfermented milk-based product	28
Bartram *et al*[15], 1994	Germany	Cross-over	12	OATT, radiopaque markers	*B. longum*	> 0.5	Active: Yogurt with 2.5 g lactulose + probiotic Control: Yogurt	21
Bazzocchi *et al*[25], 2014	Italy	Parallel groups	19:12	TITT, radiopaque markers	*L. plantarum*, *L. acidophilus*, *L. rhamnosus*, *B. longum*, *B. breve*	-	Active: Sachet with psyllium+probiotic Control: Sachet with 2.8 g maltodextrin	56
Bouvier *et al*[16], 2001	France	Parallel groups	36:36	CTT, radiopaque markers	*B. lactis* DN-173 010	97.5	Active: Probiotic fermented milk Control: Heat-treated probiotic fermented milk	11
Holma *et al*[17], 2010	Finland	Parallel groups	12:10	TITT, radiopaque markers	*L. rhamnosus* GG	20	Active: Buttermilk + probiotic and white wheat bread Control: White wheat bread	21
Hongisto *et al*[18], 2006	Finland	Parallel groups	16:14	TITT, radiopaque markers	*L. rhamnosus* GG	15	Active: Yogurt + probiotic and low fiber toast Control: Low fiber toast	21
Krammer *et al*[24], 2011	Germany	Parallel groups	12:12	CTT, radiopaque markers	*L. casei* Shirota	6.5	Active: Probiotic fermented milk drink Control: Nonfermented milk drink	28
Magro *et al*[26], 2014	Brazil	Parallel groups	26:21	CTT, radiopaque markers	*L. acidophilus* NCFM, *B. lactis* HN019	2	Active: Yogurt + polydextrose + probiotic Control: Yogurt	14
Malpeli *et al*[19], 2012	Argentina	Cross-over	83	OCTT, carmine red dye	*B. lactis* BB12	2-20	Active: Yogurt with 0.625 g inulin and oligofructose + probiotic	15
					L. casei CRL 431	2-12	Control: Yogurt	
Marteau *et al*[20], 2002	France	Cross-over	32	CTT, radiopaque markers	*B. lactis* DN-173 010	18.75	Active: Yogurt + probiotic Control: Yogurt	10
Merenstein *et al*[27], 2014	United States	Crossover	68	CTT, radiopaque markers	*B. animalis ssp. lactis Bf-6*	20-56	Active: Yogurt + probiotic Control: Yogurt	14
Rosenfeldt *et al*[21], 2003a	Denmark	Cross-over	13	GTT, radiopaque markers	*L. rhamnosus* 19070-2	20	Active: Freeze-dried powder + probiotic	18
					L. reuteri DSM 12246	20	Control: Skimmed milk powder w/dextrose	
Rosenfeldt *et al*[21], 2003b	Denmark	Cross-over	13	GTT, radiopaque markers	*L. casei* subsp. alactus CHCC 3137	20	Active: Freeze-dried powder + probiotic	18
					L. delbrueckii subsp. lactis CHCC 2329	20	Control: Skimmed milk powder w/dextrose	
					L. rhamnosus GG	20		
Sairanen *et al*[22], 2007	Finland	Parallel groups	22:20	CTT, radiopaque markers	*B. longum* BB536, *B. lactis* 420	2.4-18[1]	Active: Probiotic fermented milk	21
					L. acidophilus 145	0.48	Control: Fermented milk	
Tulk *et al*[28], 2013	Canada	Crossover	65	GTT, carmine red/carbon black capsules	*B. lactis* Bb12, *L. acidophilus* La5, *L. casei* CRL431	2	Active: Yogurt + probiotic + inulin Control: Yogurt	15
Waller *et al*[23], 2011a	United States	Parallel groups	33:34	WGTT; radiopaque markers	*B. lactis* HN019	1.8	Active: Capsule, maltodextrin, probiotic Control: Capsule, maltodextrin	14
Waller *et al*[23], 2011b	United States	Parallel groups	33:34	WGTT; radiopaque markers	*B. lactis* HN019	17.2	Active: Capsule, maltodextrin, probiotic Control: Capsule, maltodextrin	14

[1]Represents the reported range of total Bifidobacterium. CFU: Colony-forming units; CTT: Colonic transit time; GTT: Gastrointestinal transit time; OATT: Oro-anal transit time; OCTT: Oro-cecal TT; TITT: Total intestinal transit time; WGTT: Whole gut transit time.

Table 3 Subject characteristics

Study	Mean age (yr)	Female gender (%)	Mean BMI (kg/m^2)	Condition
Agrawal *et al*[14], 2009	40	100	25	IBS-C
Bartram *et al*[15], 1994	23	58	-[2]	None
Bazzocchi *et al*[25], 2014	40	86	19	Constipation
Bouvier *et al*[16], 2001	33	50	22	None
Holma *et al*[17], 2010	44	92[1]	24	Constipation
Hongisto *et al*[18], 2006	43	100	24	Constipation
Krammer *et al*[24], 2011	50	100	-[2]	Constipation
Magro *et al*[26], 2014	32	91	28	Constipation
Malpeli *et al*[19], 2012	41	100	-[2]	Constipation
Marteau *et al*[20], 2002	27	100	21	None
Merenstein *et al*[27], 2014	29	100	23	None
Rosenfeldt *et al*[21], 2003a	25	0	-[2]	None
Rosenfeldt *et al*[21], 2003b	25	0	-[2]	None
Sairanen *et al*[22], 2007	39	64	25	None
Tulk *et al*[28], 2013	29	60	24	None
Waller *et al*[23], 2011a	44	65	31	Constipation
Waller *et al*[23], 2011b	44	65	32	Constipation

[1]Percentage estimated from larger study cohort; [2]Represents missing data. BMI: Body mass index; IBS-C: Irritable bowel syndrome, constipation predominant.

Table 4 Assessment of study quality

Study	Jadad scale			
	Randomization range: 0-2	Double blinding range: 0-2	Subject account range: 0-1	Total score[1] range: 0-5
Agrawal *et al*[14], 2009	1	2	1	4
Bartram *et al*[15], 1994	1	2	0	3
Bazzocchi *et al*[25], 2014	1	2	1	4
Bouvier *et al*[16], 2001	1	2	0	3
Holma *et al*[17], 2010	1	0	1	2
Hongisto *et al*[18], 2006	1	0	0	1
Krammer *et al*[24], 2011	1	1	1	3
Magro *et al*[26], 2014	2	2	1	5
Malpeli *et al*[19], 2012	0	2	1	3
Marteau *et al*[20], 2002	1	2	1	4
Merenstein *et al*[27], 2014	2	2	1	5
Rosenfeldt *et al*[21], 2003a	1	1	0	2
Rosenfeldt *et al*[21], 2003b	1	1	0	2
Sairanen *et al*[22], 2007	1	1	0	2
Tulk *et al*[28], 2013	1	1	1	3
Waller *et al*[23], 2011a	2	2	1	5
Waller *et al*[23], 2011b	2	2	1	5

[1]Higher scores represent better study quality.

Table 5 Subgroup analysis of study- and subject-related factors on intestinal transit time

Study	SMD	95%CI	*P* value (pre-post)	*P* value (between groups)
Subject condition				
Constipation/IBS-C (n = 9)	0.57	0.39-0.75	< 0.001	< 0.01
Healthy (n = 8)	0.22	0.05-0.39	0.01	
Study quality				
Jadad score ⩾ 3 (n = 12)	0.45	0.31-0.59	< 0.001	0.01
Jadad score < 3 (n = 5)	0.00	-0.33-0.33	> 0.99	
Age[1]				
⩾ 39 yr (n = 9)	0.51	0.29-0.73	< 0.001	0.08
< 39 yr (n = 8)	0.27	0.09-0.44	< 0.01	
Publication year				
After 2008 (n = 10)	0.47	0.29-0.65	< 0.001	0.08
Before 2008 (n = 7)	0.20	-0.03-0.44	0.09	
Number of probiotic strains				
Single strain (n = 10)	0.49	0.32-0.66	< 0.001	0.09
Multiple strains (n = 7)	0.23	-0.01-0.47	0.06	
Study design				
Parallel groups (n = 11)	0.48	0.31-0.65	< 0.001	0.09
Cross-over (n = 6)	0.26	-0.02-0.46	0.07	
Body mass index[1,2]				
⩾ 25 kg/m^2 (n = 5)	0.59	0.24-0.94	< 0.001	0.16
< 25 kg/m^2 (n = 7)	0.31	0.13-0.49	< 0.001	
Treatment duration[1]				
< 18 d (n = 8)	0.45	0.29-0.60	< 0.001	0.17
⩾ 18 d (n = 9)	0.22	-0.06-0.50	0.12	
Geographic location				
Americas (n = 6)	0.47	0.26-0.67	< 0.001	0.20
Europe (n = 11)	0.28	0.07-0.49	< 0.01	
Female gender proportion[1]				
⩾ 86% (n = 9)	0.47	0.30-0.64	< 0.01	0.22
< 86% (n = 8)	0.27	0.00-0.54	< 0.05	
Confounding treatments[3]				
Yes (n = 7)	0.46	0.24-0.67	< 0.001	0.32
No (n = 10)	0.30	0.10-0.51	< 0.01	
Daily probiotic dosage[1]				
⩾ 1.6^{10} CFU (n = 8)	0.40	0.12-0.67	< 0.01	0.74
< 1.6^{10} CFU (n = 7)	0.34	0.16-0.52	< 0.001	

[1]Categorized by median value; [2]Body mass index not reported for 5 treatment effects; [3]Includes studies where treatment included probiotics plus fiber or non-digestible sugar. Variables sorted from lowest to highest between-groups *P* value; n represents the number of treatment effects. IBS-C: Irritable bowel syndrome, constipation predominant; SMD: Standardized mean difference.

(MR) (Table 6) were performed to determine the influence of study- and subject-related characteristics on ITT. Probiotic supplementation reduced ITT in comparison to controls in several of the analyzed subgroups. Greater reductions in ITT were observed with probiotics in subjects with *vs* without constipation (SA and MR, $P < 0.01$) and in high-quality (Jadad score ⩾ 3) *vs* low-quality (Jadad score < 3) studies (SA and MR, $P = 0.01$). There were trends for greater probiotic efficacy with older age (SA, $P = 0.08$, MR, $P = 0.02$), in recently published studies (SA, $P = 0.08$), with parallel groups study designs (SA, $P = 0.08$), higher percentage of female subjects (SA, $P = 0.08$,

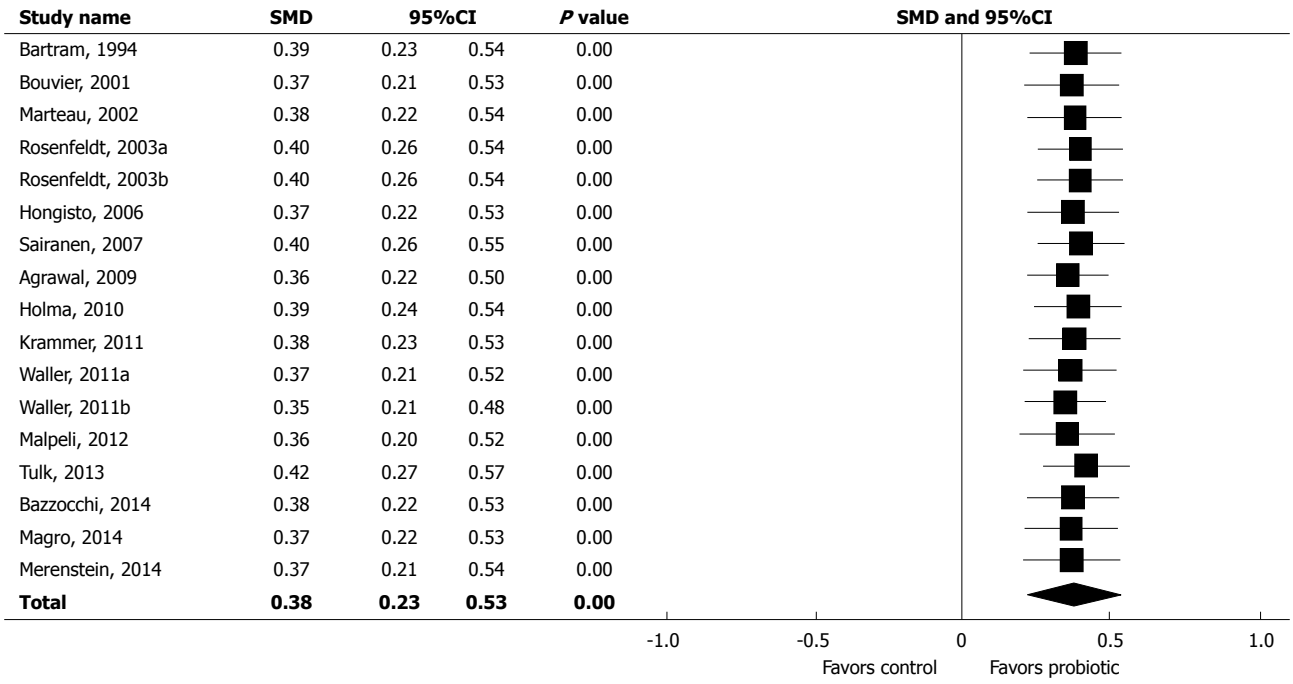

Figure 4 One study removed forest plot of standardized mean difference in intestinal transit time across studies. SMD: Standardized mean difference.

Table 6 Meta-regression of study- and subject-related factors on intestinal transit time

Variable	Unit of measure	Intercept	Point estimate	Explained variance (%)	*P* value
Constipation/ IBS-C	1 = Yes; 0 = No	0.218	0.352	38	< 0.01
Jadad score	Per 1 unit	-0.117	0.141	31	0.01
Age	Per 1 yr	-0.352	0.021	27	0.02
Female gender proportion	Per 10%	-0.045	0.055	26	0.02
Number of probiotic strains	Per 1 strain	0.618	-0.133	20	< 0.05
Body mass index[1]	Per 1 kg/m^2	-0.526	0.037	22	0.08
Treatment duration	Per 1 d	0.392	-0.004	0	0.96
Daily probiotic dosage	Per 10 × 10^9 CFU	0.385	-0.001	0	0.98

[1]Body mass index not reported for 5 treatment effects. Variables sorted from greatest to least explained variance.

Table 7 Subgroup analysis of probiotic strains on intestinal transit time

Probiotic strain	No. of treatment effects	SMD	95%CI	*P* value
B. lactis HN019	3	0.67	0.37-0.97	< 0.001
B. lactis DN-173 010	3	0.54	0.16-0.92	< 0.01
L. casei CRL 431	2	0.33	-0.10-0.75	0.14
B. lactis BB12	2	0.33	-0.10-0.75	0.14
L. rhamnosus GG	3	0.10	-0.35-0.55	0.67

Probiotic strains sorted from highest to lowest standard mean difference. SMD: Standardized mean difference.

MR, $P = 0.02$), single-strain probiotics (SA, $P = 0.09$, MR, $P < 0.05$) and higher body mass index (SA, $P = 0.16$, MR, $P = 0.08$). Treatment duration, geographic location of study, inclusion of potentially confounding treatments, and daily probiotic dosage were not found to have a significant influence on probiotic efficacy in subgroup analysis and meta-regression. Analysis of outcomes by probiotic strain identified medium to large treatment effects with *B. lactis* HN019 (SMD: 0.67, $P < 0.001$) and *B. lactis* DN-173 010 (SMD: 0.54, $P < 0.01$) while treatment effects with other strains were small (SMD: 0.10-0.33) and not statistically significant (Table 7).

DISCUSSION

An ever-increasing body of evidence implicates the gastrointestinal microbiome in defining states of health and disease[29]. Probiotics may restore the composition of the gut microbiome and support beneficial functions to gut microbial communities, resulting in amelioration of gut inflammation and other disease phenotypes[30]. Consequently, probiotic supplementation is increasingly touted as an effective and accessible means of improving gut health, even in the general population of healthy adults. The current systematic review and meta-analysis demonstrates that short-term probiotic supplementation yielded moderate ITT reductions in adults. Additionally, the treatment effect of probiotics was greater in subjects with constipation, in high-quality studies, and with certain probiotic strains. In contrast to the moderate treatment effect observed in constipated subjects, probiotics only minimally influenced ITT in non-constipated adults. Given this finding, it appears that probiotic consumption will

not lead to undesired short ITT or diarrhea. However, probiotic consumption for the sole purpose of reducing ITT is unjustified in healthy adults. Nevertheless, this finding does not diminish other beneficial effects that have been observed with probiotics in healthy adults[31,32].

In this meta-analysis, there was a trend for greater treatment effects with probiotics in parallel groups study designs compared to crossover studies (SMD: 0.48 *vs* 0.26, $P = 0.09$). Although there is no clear explanation for this finding, data from one included study deserves further discussion. The study of Merenstein *et al*[27] enrolled 68 healthy women using a crossover design, with a 6-wk washout between treatment periods. However, a significant carry-over effect was observed at the start of the second treatment period. For purposes of this meta-analysis, we treated this study as a parallel groups design using data from the first treatment period only[33]. Although the presence of a carry-over effect was not mentioned in the other crossover studies included in this analysis, the fact that washout periods ranged from 2 to 6 wk with significant carryover identified even after 6 wk in the Merenstein study raises the question of whether carry-over effects may have influenced outcomes of other crossover studies. Although crossover studies may initially appear attractive to researchers given the smaller sample size requirements compared to parallel groups designs, we propose that crossover designs are inappropriate in probiotic clinical trials unless the washout period for the probiotic has been previously established for the specific condition under study.

In comparison to our previous meta-analysis on this topic, the treatment effect of probiotics on ITT was largely unchanged (SMD: 0.40 *vs* 0.38). Importantly, with the addition of more studies, we were able to explore potential sources of heterogeneity among studies with greater precision. Novel subgroup findings included the observation of moderate probiotic treatment effects (SMD: 0.45) in high-quality studies, but no treatment effect (SMD: 0.0) in low-quality studies. Although the treatment effect sizes in parallel groups and crossover studies remained largely unchanged, study design is now a considerably stronger predictor of heterogeneity in ITT outcomes given the inclusion of additional studies. We also identified that single-strain probiotics were more efficacious than multiple strain probiotics. Although *B. lactis* HN019 and *B. lactis* DN-173 010 remained the most efficacious probiotic strains, we were able to analyze additional probiotic strains that yielded modest improvements in ITT relative to placebo.

The strengths of this systematic review and meta-analysis are inclusion of only RCTs and a comprehensive assessment of the influence of moderator variables on ITT with probiotic supplementation. Our study also revealed several limitations in the design of ITT studies with probiotics. First, the treatment duration of included studies ranged from 10 to 56 d. Although the long-term safety of probiotics is well established[34], probiotic efficacy on ITT beyond 8 wk cannot be interpreted with the current analysis. Second, although the therapeutic benefit of probiotics appears to be strain-specific, the small number of studies performed with each strain prevented robust strain-specific comparisons. Finally, subject characteristics were relatively homogenous among studies with regard to age and gender. Therefore, the generalizability of these findings to the general population, particularly males and the elderly, is unknown. These findings give specific suggestions for future research in this field.

In conclusion, probiotic supplementation is moderately efficacious for reducing ITT in adults. Probiotics were most efficacious in constipated subjects, when evaluated in high-quality studies, and with certain probiotic strains.

COMMENTS

Background

Functional gastrointestinal disorders are common in the general population, with slow intestinal transit a common symptom. No therapy is highly efficacious, safe, and cost effective for treatment of slow-transit bowel disorders. Probiotics have been extensively studied for treatment of gastrointestinal disorders and may confer improvements in bowel regularity.

Research frontiers

Clinical trials of probiotic supplementation on intestinal transit time (ITT) yield discrepant results. The authors performed a contemporary systematic review and meta-analysis on the efficacy of probiotic supplementation on ITT in adults, with a secondary focus on exploring sources of heterogeneity through meta-regression and subgroup analyses.

Innovations and breakthroughs

Probiotics are most efficacious in constipated subjects, when evaluated in high-quality studies, and with certain probiotic strains.

Applications

Probiotic supplementation appears to confer clinically meaningful improvements in intestinal transit in subjects with constipation. Probiotic efficacy also significantly differs according to strain.

Terminology

Probiotics are live micro-organisms that confer a health benefit on the host when administered in adequate dosages. Intestinal transit time is an indicator of the time taken for a food bolus to travel through the gastrointestinal system. The standardized mean difference is a statistical measure of effect size for continuous outcomes, defined as the mean difference between groups divided by the pooled standard deviation.

Peer-review

Very nice manuscript.

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P- Reviewer: Pehl C, Thompson JR **S- Editor**: Yu J **L- Editor**: A **E- Editor**: Wang CH

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DOI: 10.3748/wjg.v22.i21.5132

World J Gastroenterol 2016 June 7; 22(21): 5132-5136
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

CASE REPORT

Modified single transluminal gateway transcystic multiple drainage technique for a huge infected walled-off pancreatic necrosis: A case report

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Kosuke Minaga, Masayuki Kitano, Hajime Imai, Kentaro Yamao, Ken Kamata, Takeshi Miyata, Tomohiko Matsuda, Shunsuke Omoto, Kumpei Kadosaka, Tomoe Yoshikawa, Masatoshi Kudo, Department of Gastroenterology and Hepatology, Kinki University Faculty of Medicine, Osaka-Sayama 589-8511, Japan

Author contributions: Minaga K contributed to manuscript writing, performing endoscopic interventions; Kitano M contributed to drafting conception and design, performing endoscopic interventions; Imai H, Yamao K, Kamata K, Miyata T, Matsuda T, Omoto S, Kadosaka K and Yoshikawa T contributed to writing the manuscript; Kudo M contributed to drafting conception and design; all authors helped to perform the research.

Supported by The Japan Society for the Promotion of Science and the Japanese Foundation for the Research and Promotion of Endoscopy, No. 22590764 and No. 25461035.

Institutional review board statement: This study was approved by the Institutional Review Board of Kinki University Faculty of Medicine.

Informed consent statement: The patient provided informed written consent prior to study enrollment.

Conflict-of-interest statement: All authors declare no conflicts-of-interest related to this article.

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Received: January 9, 2016
Peer-review started: January 10, 2016
First decision: February 18, 2016
Revised: February 24, 2016
Accepted: March 18, 2016
Article in press: March 18, 2016
Published online: June 7, 2016

Abstract

We report a successful endoscopic ultrasonography-guided drainage of a huge infected multilocular walled-off necrosis (WON) that was treated by a modified single transluminal gateway transcystic multiple drainage (SGTMD) technique. After placing a wide-caliber fully covered metal stent, follow-up computed tomography revealed an undrained subcavity of WON. A large fistula that was created by the wide-caliber metal stent enabled the insertion of a forward-viewing upper endoscope directly into the main cavity, and the narrow connection route within the main cavity to the subcavity was identified with a direct view, leading to the successful drainage of the subcavity. This modified SGTMD technique appears to be useful for seeking connection routes between subcavities of WON in some cases.

Key words: Endoscopic ultrasonography; Infected pancreatic necrosis; Walled-off necrosis; Endoscopic ultrasonography-guided drainage; Acute pancreatitis

Core tip: Walled-off necrosis (WON) remains difficult to endoscopically manage because of insufficient drainage of solid necrotic tissues. Here, we present a case of successful drainage of a huge WON *via* a modified single transluminal gateway transcystic multiple drainage technique. After placing a wide-caliber covered metal stent, follow-up computed tomography revealed an undrained subcavity of WON. A large fistula created by the metal stent enabled the insertion of an upper endoscope directly into the main cavity, and the narrow connection route within the main cavity to the subcavity was identified with a direct view, leading to the successful drainage of the subcavity.

Minaga K, Kitano M, Imai H, Yamao K, Kamata K, Miyata T, Matsuda T, Omoto S, Kadosaka K, Yoshikawa T, Kudo M. Modified single transluminal gateway transcystic multiple drainage technique for a huge infected walled-off pancreatic necrosis: A case report. *World J Gastroenterol* 2016; 22(21): 5132-5136 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i21/5132.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i21.5132

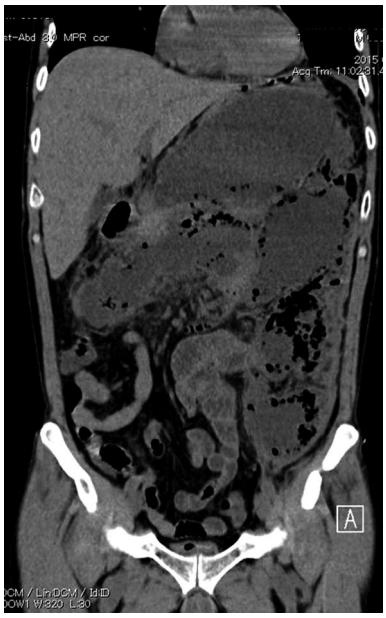

Figure 1 Abdominal computed tomography scan showing a huge multilocular walled-off necrosis replacing the body and tail of the pancreas, which extended to the pelvis. Gas bubbles were observed in the cavity.

INTRODUCTION

Endoscopic ultrasonography (EUS)-guided drainage for pancreatic fluid collection (PFC) is increasingly used as a minimally invasive alternative to surgical and percutaneous drainage[1-3]. However, walled-off necrosis (WON) remains difficult to endoscopically manage because of insufficient drainage of solid necrotic tissues. Various techniques, such as the use of wide-caliber metal stents[4,5], direct endoscopic necrosectomy[6,7] and multiple transluminal gateway technique[8] are reportedly useful for managing WON. However, responses to these advanced techniques remain unsatisfactory in some cases. Recently, a single transluminal gateway transcystic multiple drainage (SGTMD) was developed for treating complicated multilocular WON[9]. Here, we present a case of successful endoscopic drainage of a huge infected multilocular WON *via* a modified SGTMD technique.

CASE REPORT

A 49-year-old male presented with upper abdominal pain and high fever of 7 d duration. He was diagnosed with alcohol-induced severe acute pancreatitis 1 mo before and was discharged 6 d after admission from a neighbouring general hospital. His computed tomography (CT) severity index[10] was 6. He was re-admitted to our hospital with the above-mentioned chief complaints. Laboratory tests revealed elevated C-reactive protein (CRP) and procalcitonin levels (27.8 mg/dL and 6.17 ng/mL, respectively). Elevated levels of kidney function parameters were also noted (blood urea nitrogen level, 77 mg/dL; serum creatinine level, 3.14 mg/dL). An abdominal CT revealed a huge multilocular WON measuring 31 cm × 16 cm, which spread from the pancreas to pelvis (Figure 1). Clinically, infection of the necrosis was assumed. Doripenem was intravenously introduced; however, his clinical symptoms and elevated inflammatory reaction persisted. As the main cavity of WON was close to the gastric lumen, we decided to puncture WON under EUS guidance. EUS-guided transluminal drainage was performed; a wide-caliber fully covered TTS Niti-S esophageal stent (internal diameter, 16 mm; maximum flange diameter, 24 mm; length, 40 mm; Taewoong Medical, Seoul, South Korea) was placed (Figure 2). Through the metal stent, a 7-Fr double-pigtail plastic stent (length, 80 mm) and a 7-Fr nasocystic catheter were inserted (Figure 3). During the procedure, approximately 2.4 L of purulent fluid were suctioned. A follow-up abdominal CT obtained 1 wk after the procedure demonstrated a significant reduction in the size of the main cavity; however, the undrained subcavity remained, which was mainly located in the left anterior pararenal space and extended to the left pelvis (Figure 4). Additional drainage targeting the subcavity was required because high fever continued after the procedure. Because the subcavity was not adjacent to the stomach or duodenum, additional EUS-guided puncture was difficult. CT suggested communication between the subcavity and main cavity; therefore, a SGTMD procedure was considered. Repeated attempts to determine the connection route within the main cavity to the subcavity using an ERCP catheter and 0.025-inch guidewire were unsuccessful. The metal stent was removed, and a large fistula that was created by the metal stent enabled the insertion

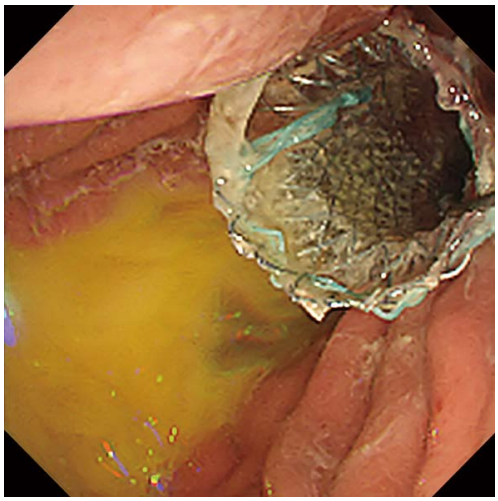

Figure 2 Successful deployment of a wide-caliber fully covered TTS Niti-S esophageal stent. Purulent fluid was observed in the gastric lumen.

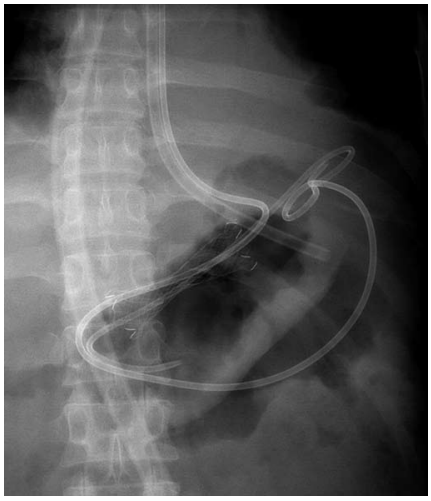

Figure 3 A 7-Fr double-pigtail plastic stent and a 7-Fr nasocystic catheter were deployed through the fully covered metal stent.

of a forward-viewing upper endoscope directly into the main cavity. After the endoscope was advanced into the cavity, a narrow connection route was identified (Figure 5). Contrast medium was injected into the connection. Having confirmed the detection of the subcavity, the guidewire was inserted into the cavity and two 7-Fr double-pigtail plastic stents (lengths, 120 and 80 mm, respectively) were deployed (Figure 6). No procedure-related complications were observed. After additional endoscopic management, high fever resolved over the course of a few days and CRP levels significantly decreased. CT revealed that the subcavity of WON was well drained. The patient completely recovered and was discharged after 3 wk of hospitalization. Follow-up CT obtained 1 month after discharge revealed that WON had mostly collapsed (Figure 7) and the patient remained symptom free.

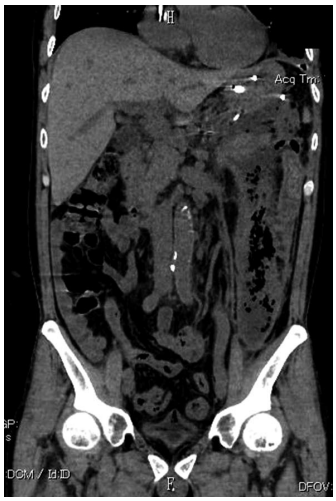

Figure 4 Computed tomography one week after initial drainage showed an undrained subcavity, located mainly at the left anterior pararenal space that extended to the left pelvis.

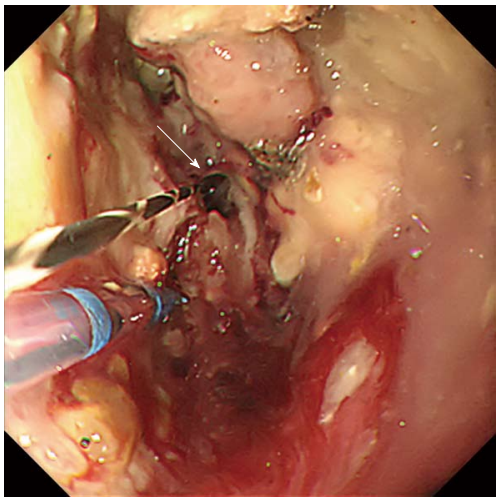

Figure 5 Endoscopic view of the cavity of walled-off necrosis by a modified single transluminal gateway transcystic multiple drainage technique. An upper endoscope was inserted into the walled-off necrosis (WON) through the fistula and a narrow connection route within the main cavity to the subcavity could be identified directly (white arrow).

DISCUSSION

Over the last decade, techniques for pancreatic fluid collection have shifted toward minimally invasive approaches. Since first reported in 1992 by Grimm *et al*[1] EUS-guided transluminal drainage for pancreatic fluid collection has played a pivotal role and spread worldwide as a minimally invasive alternative to surgical and percutaneous drainage[1-3]. However, the clinical response rate of the conventional single transluminal gateway technique deploying single or multiple stenting for treating WON is not satisfactory (described as 45%-63%)[8,11]. Recently, various techniques, such as the use of wide-caliber metal

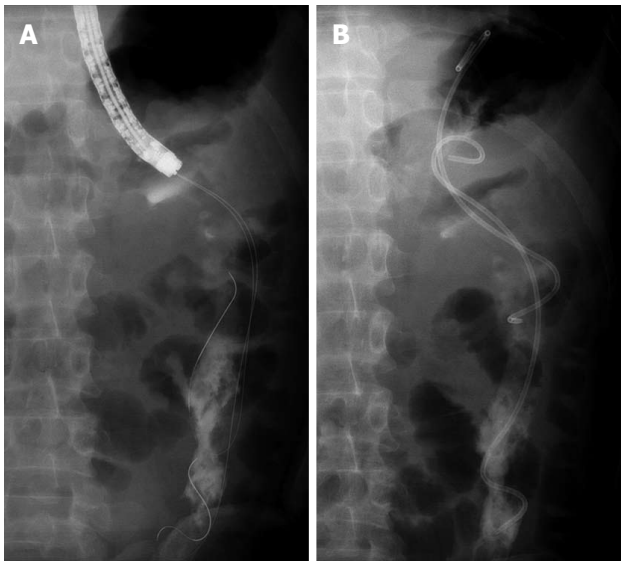

Figure 6 Fluoroscopic view of modified single transluminal gateway transcystic multiple drainage technique. With a direct view of the connection route, a 0.025-inch guidewire was inserted into the subcavity (A) and two 7-Fr double-pigtail plastic stents were deployed (B).

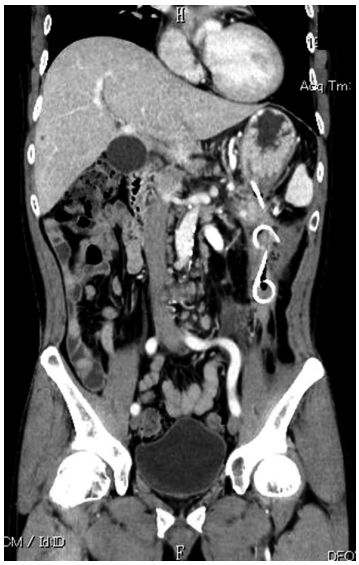

Figure 7 Follow-up computed tomography obtained one month after discharge revealed the WON had mostly collapsed.

stents[4,5], direct endoscopic necrosectomy[6,7] and multiple transluminal gateway technique[8] have improved the clinical success rate of endoscopic management of WON. However, response to these advanced techniques remains unsatisfactory in some cases. Mukai *et al*[9] recently described a novel SGTMD procedure for complicated multilocular WON and reported successful drainage in five cases using this technique. When subcavities are located far from the gastrointestinal lumen, percutaneous approach would have been used conventionally. Mukai *et al*[9] hypothesized that the multilocular cavity may have originally been unilocular and separated into subcavities with tiny, narrow connections during the process of treatment and collapse. They used an ERCP catheter and soft guidewire to locate tiny, narrow connections. In this case, we repeatedly attempted to identify the connection using an ERCP catheter and soft guidewire through the metal stent under fluoroscopic guidance, but the guidewire curled up in the main cavity and failed to locate a connection route. Instead, we inserted the upper endoscope into the cavity through the large fistula, which enabled the narrow connection route to be directly observed. The guidewire was easily and safely advanced into the subcavity, and successful drainage of the subcavity was achieved. This is a modified technique of the previously described SGTMD. In addition to SGTMD, having a direct view to identify the connection route may lead to a higher success rate in some cases.

In this case, the pig-tail stents have been left in place during 6 mo follow-up. This is because the previous studies revealed that stent retrieval was associated with higher PFC recurrence rates[12,13].

In conclusion, we presented a case of successful endoscopic drainage of a huge infected multilocular WON by a modified SGTMD technique with direct endoscope insertion into the cavity. This modified SGTMD technique appears to be useful in seeking connection routes between the subcavities of WON and might avoid the requirement for a more invasive drainage procedure, such as endoscopic or surgical necrosectomy.

COMMENTS

Case characteristics

One month after being diagnosed with alcohol-induced severe acute pancreatitis, a 49-year-old male presented with upper abdominal pain and high fever of 7 d duration.

Clinical diagnosis

The patient had upper abdominal pain and high fever.

Differential diagnosis

Pancreatic pseudocyst.

Laboratory diagnosis

The laboratory findings showed elevated C-reactive protein, procalcitonin levels and renal dysfunction.

Imaging diagnosis

Abdominal computed tomography demonstrated a huge multilocular WON measuring 31 cm × 16 cm, which spread from the pancreas to pelvis.

Pathological diagnosis

Pathological examination was not performed in this case.

Treatment

Endoscopic drainage with a modified single transluminal gateway transcystic multiple drainage (SGTMD) technique was performed.

Related reports

WON remains difficult to endoscopically manage because of insufficient drainage of solid necrotic tissues. Various techniques, such as the use of wide-caliber metal stents, direct endoscopic necrosectomy, multiple transluminal gateway technique and SGTMD technique were developed for treating WON.

Term explanation

Modified SGTMD is a novel alternative technique for drainage of WON which means a single transluminal gateway transcystic multiple drainage with direct endoscope insertion into the cavity.

Experiences and lessons

Modified SGTMD technique appears to be useful in seeking connection routes between the subcavities of WON and might avoid the requirement for a more invasive drainage procedure, such as endoscopic or surgical necrosectomy.

Peer-review

This case report is interesting and well documented.

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P- Reviewer: Wronski M **S- Editor**: Qi Y **L- Editor**: A
E- Editor: Zhang DN

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ISSN 1007-9327