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

World Journal of *Gastroenterology*

World J Gastroenterol 2016 October 28; 22(40): 8853-9038

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 40 October 28, 2016

LETTERS TO THE EDITOR

World Journal of Gastroenterology
Volume 22 Number 40 October 28, 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

EDITORIAL OFFICE
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World Journal of Gastroenterology
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8226 Regency Drive,
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E-mail: editorialoffice@wjgnet.com
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
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PUBLISHER
Baishideng Publishing Group Inc
8226 Regency Drive,
Pleasanton, CA 94588, USA
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PUBLICATION DATE
October 28, 2016

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DOI: 10.3748/wjg.v22.i40.8853

World J Gastroenterol 2016 October 28; 22(40): 8853-8861
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

EDITORIAL

Transarterial chemoembolization with drug-eluting beads in hepatocellular carcinoma

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Author contributions: This manuscript was written completely by the stated authors.

Conflict-of-interest statement: There is no conflict of interest.

Manuscript source: Invited manuscript

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Received: July 13, 2016
Peer-review started: July 18, 2016
First decision: August 8, 2016
Revised: August 25, 2016
Accepted: September 14, 2016
Article in press: September 14, 2016
Published online: October 28, 2016

Abstract

Transarterial chemoembolization (TACE) is a widely used standard treatment for patients with hepatocellular carcinoma (HCC) who are not suitable candidates for curative treatments. The rationale for TACE is that intra-arterial chemotherapy using lipiodol and chemotherapeutic agents, followed by selective vascular embolization, results in a strong cytotoxic effect as well as ischemia (conventional TACE). Recently, drug-eluting beads (DC Beads®) have been developed for transcatheter treatment of HCC to deliver higher doses of the chemotherapeutic agent and to prolong contact time with the tumor. DC Beads® can actively sequester doxorubicin hydrochloride from solution and release it in a controlled sustained fashion. Treatment with DC Beads® substantially reduced the amount of chemotherapeutic agent that reached the systemic circulation compared with conventional, lipiodol-based regimens, significantly reducing drug-related adverse events. In this article, we describe the treatment response, survival, and safety of TACE used with drug-eluting beads for the treatment of HCC and discuss future therapeutic possibilities.

Key words: Hepatocellular carcinoma; Transarterial chemoembolization; Conventional TACE; Drug-eluting beads; Treatment response

Core tip: Drug eluting beads are relatively new embolic agents that allow sustained release of chemotherapeutic agents in a localized fashion to the tumor. The advantage of DC bead transarterial chemoembolization (TACE) is a better combined ischemic and cytotoxic effect locally and less system toxicity when compared with conventional TACE.

Nam HC, Jang B, Song MJ. Transarterial chemoembolization with drug-eluting beads in hepatocellular carcinoma. *World J Gastroenterol* 2016; 22(40): 8853-8861 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i40/8853.htm DOI:

http://dx.doi.org/10.3748/wjg.v22.i40.8853

INTRODUCTION

Hepatocellular carcinoma (HCC) is the most common liver malignancy worldwide[1,2]. Unlike other malignancies, the incidence and mortality rates of HCC will continue to increase through over the next 20 years in some countries[3,4]. The incidence of HCC is influenced by major risk factors that include hepatitis B virus, hepatitis C virus, alcohol consumption and nonalcoholic steatohepatitis. The majority of patients with HCC present with advanced disease at the time of diagnosis and are not candidates for curative therapies such as surgery, transplantation, or radiofrequency ablation[5].

Transarterial chemoembolization (TACE) is the most commonly used as palliative treatment for patients with unresectable HCC[6,7]. The principle of conventional TACE (cTACE) is the synergistic effect of cytotoxic chemotherapy and ischemia. Intra-arterial chemotherapeutic agents mixed with lipiodol cause cytotoxic damage in tumor cells, and embolization of feeding vessels by gelatine or Gelform particles results in ischemia[8]. In a meta-analysis, Llovet *et al*[9] reported that TACE results in a significantly higher survival rate compared with best supportive care in well-selected cases.

From a pharmacokinetics perspective, for the best effect, higher doses of the intra-arterial chemotherapeutic agent should be retained within the tumor. Furthermore, a chemotherapeutic drug that is released can reduce systemic side effects. Drug-eluting beads had been developed with these objectives in mind[10]. DC Beads® (Biocompatibles, Surrey, United Kingdom) can load and release doxorubicin hydrochloride in a controlled manner[11]. TACE used with beads loaded with doxorubicin (DEBDOX) induced significantly fewer drug-related side effects compared with cTACE[10,12].

Several studies have shown promising outcomes of TACE with DEBDOX for intermediate-stage HCC, and this could be an alternative to cTACE. Here, we summarize recent results for the use of TACE with drug-eluting beads in terms of the treatment response, survival, and adverse events and discuss future therapeutic possibilities.

TECHNICAL ASPECT AND CHARACTERISTICS OF DC BEADS

There is no standard method for conducting cTACE. The procedure involves local infusion of chemotherapeutic agents with selective embolization of the HCC feeding arteries; however, the selection of the chemotherapeutic agent contrast agent, and embolization material vary from center to center and country to country[13]. From a technical perspective, cTACE should be as selective as possible, and further standardization of cTACE protocols is needed[14]. Inconsistency with using the technique is the major obstacle to standardization of the cTACE procedure. Potentially, TACE with DEBDOX could maintain sufficient consistency and repeatability during the procedure to allow its use as a standard treatment for HCC.

DC Beads are hydrogel microspheres that are biocompatible, hydrophilic, non-resorbable, precisely calibrated, and capable of loading chemotherapeutic agents. DC Beads are produced from a polyvinyl alcohol hydrogel that has been modified with sulfonate groups for the controlled loading and delivery of chemotherapeutic drugs, such as doxorubicin and irinotecan[11,15]. Positively charged doxorubicin HCl is drawn to the negatively charged sulfonate groups of the DC beads spheres by an ion-exchange mechanism. After mixing the doxorubicin, DC beads suspension, and contrast agent, only 0.2% of the doxorubicin remains free in the systemic circulation. The low rate of doxorubicin release could minimize the systemic side effects of TACE with DEBDOX compared with cTACE[12,16]. The doxorubicin is gradually sequestered inside the tumor because the drug dissociates from the DC beads only under specific ionic circumstances such as those found in tumor cells.

DOXORUBICIN LOADING AND PLANNED DOSING

DC Beads microspheres are packaged in 2 mL vials of hydrated beads in sodium phosphate solution. Each vial can be loaded with 50-75 mg doxorubicin. The maximum dose for a single treatment has been set at 150 mg, based on a study showing that this was the maximum dose for systemic infusion of doxorubicin[10]. The dose of doxorubicin should depend on the tumor burden. However, absolute recommendations are difficult to determine due to individual patient- and tumor-related factors. Expert consensus hold that the doxorubicin dose should be varied based on the tumor status defined by the Milan criteria for liver transplantation (a solitary tumor $\leqslant$ 5 cm or a maximum of three tumors all $\leqslant$ 3 cm)[14].

For limited disease (within the Milan criteria), the treatment strategy needs to include escalation of the doxorubicin dose up to 75 mg per single TACE. For advanced disease (exceeding the Milan criteria), the doxorubicin dose could be increased to a maximum of 150 mg[14,17].

SELECTION OF THE BEADS DIAMETER

The DC Beads come in four sizes: 100-300, 300-500, 500-700, and 700-900 μm. HCC with an arteriovenous shunt (AV) confers an increased risk of pulmonary complications during TACE[18]. Therefore, the bead size should be chosen carefully, especially for patients with an AV shunt.

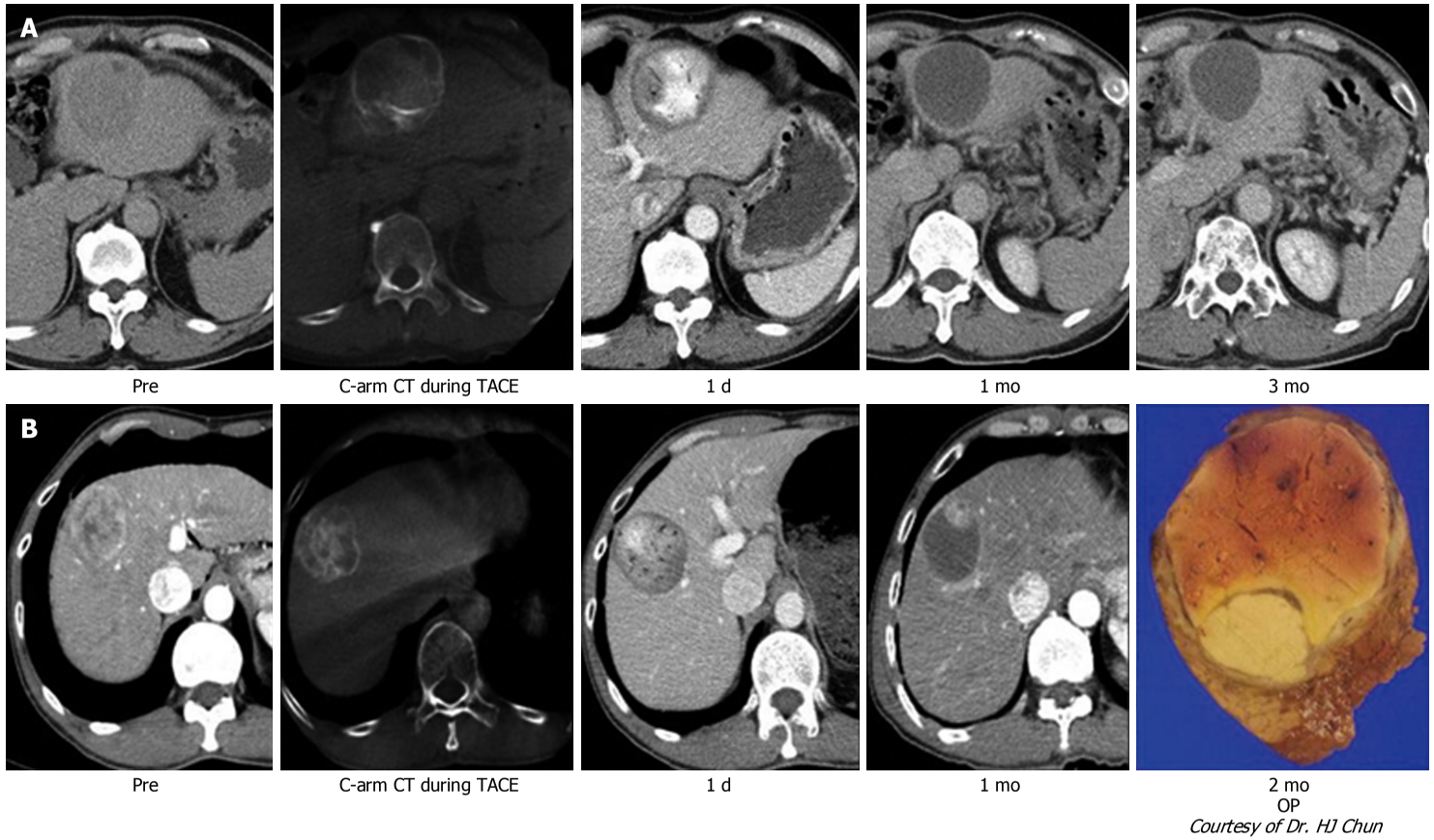

Figure 1 Typical imaging finding after transarterial chemoembolization with drug-eluting bead. A: Nodular hepatocellular carcinoma (HCC) with arterial enhancement showed the total necrosis of HCC through follow-up computed tomography (CT) imaging; B: Nodular HCC showed nodular arterial enhancing viable portion within the partial necrosis of HCC in follow-up CT imaging. After operation, viable HCC in resected HCC showed the matched lesion in the CT imaging.

Histological analysis of HCC explants treated with DC Beads showed that 100-300 μm beads are distributed both within and at the periphery of the tumor[19]. Lewis *et al*[13] showed that 100-300 μm beads triggered broad necrosis of the tumor and adjacent peritumoral hepatic tissue without causing vasculopathy or inflammation[20]. As shown in Figure 1, the cytotoxic agent and ischemia induce profound liquefaction and necrosis of the tumor.

In comparison, DC Beads with larger diameters (700-900 μm) induce limited necrosis compared with smaller beads[21]. Beads with smaller diameters can be delivered to distal vessels where they obstruct collateral channels, while, larger beads occlude more proximal vessels, allowing blood to be supplied to the tumor *via* collateral vessels, which are not occluded by the beads[17]. Recently, the diameters of drug-eluting microspheres are smaller than that of DC Beads are introduced in some studies[22-24]. The diameter of each microsphere is as follows: HepaSphere; 30-60 μm[22], M1 DC Beads; 70-150 μm[23], TANDEM; 40-100 μm[24], respectively.

INDICATIONS

The indications for DC Beads use with TACE (DC Beads TACE) are similar to those for cTACE. DC Beads TACE may be a better option, particularly in patients with more advanced liver disease (Child-Pugh B, Eastern Cooperative Oncology Group (ECOG) 2, Barcelona Clinic Liver Cancer (BCLC) C, bilobar, or recurrent disease) or patients with mild to moderate cardiac failure[25].

RESPONSE TO DC BEADS TACE

Varela *et al*[10] reported a 75% overall response rate to TACE with DEBDOX in a phase Ⅰ/Ⅱ clinical trial that included tumors with a mean diameter of 4.6 cm, and the majority of patients were Okuda Ⅰ (Okuda Ⅰ/Ⅱ: 26/1). In another study, Malagari *et al*[16] reported a complete response (CR) was accomplished in 15.5% of 71 patients (mean tumor size 6.2 cm) who underwent DC Beads TACE. Furthermore, a 66.2%-85.5% objective response (OR) rate was achieved after an additional four cycles of treatment in the same study group. Poon *et al*[12] reported the treatment responses of patients with multiple larger HCCs, in whom the mean number of tumors was 3 ± 3, the maximum tumor size was 7.6 ± 4.8 cm, and the summed tumor size was 10.0 ± 5.8 cm. While treatment response rates of 16%-35% have been reported in past cTACE studies[26,27], a favorable treatment response was seen in this larger HCC group; the OR rate 1 mo after DC Beads TACE was 70% [CR = 6.7%; partial response (PR) 63.3%] using the modified Response Evaluation Criteria in Solid Tumors[12].

The PRECISION V prospective clinical trial reported a 6-mo OR rate of 52%, which is comparable to the rates of 44%-82% seen in previous phase Ⅰ/Ⅱ studies[24]. Although statistical significance was not achieved in terms of superior OR rates for DC Beads TACE over cTACE, the formed seemed to be associated with higher response rates in terms of CR, OR, and disease control. Moreover, DC Beads proved to be effective in those with advanced disease as shown by the improved treatment response and disease control with acceptable safety profiles. This may provide a niche for those with poorer conditions, such as patients with Child-Pugh B and ECOG 1 disease, for whom the effect of conventional treatment has been minimal.

In an Asian case-control study, Song *et al*[28] reported that larger tumors (> 5 cm) or multiple HCCs showed a better treatment response to DC Beads TACE than to cTACE. Another comparative study showed that the OR rate of the DC Beads group was significantly better than that of the cTACE group (81.6% *vs* 49.2%, $P < 0.001$). A subgroup analysis confirmed that intermediate-stage HCC had a significantly higher OR and time to progression when treated with DC Beads TACE compared with cTACE (75.7% *vs* 34.1%, $P < 0.001$; 11.7 *vs* 7.6 mo, $P = 0.018$, respectively). In terms of liver toxicity, the DC Beads group did not differ significantly from the cTACE group ($P > 0.05$)[29]. This lack of significance may be ascribable to the heterogeneous ethnicities of the patient population and various etiologies of the underlying liver disease. This suggests the importance of strict patient selection with considerate and delicate angiographic techniques when conducting TACE.

However, these significant improvements in treatment responses of DC Beads TACE as compared to those of cTACE were not proven in other studies. In two randomized controlled trials (RCTs), Golfieri *et al*[30] reported treatment response of 177 HCC patients involving 89 in DC Beads TACE and 88 in cTACE. OR rates at 3 mo showed 74.7% and 74.1% for DC Beads TACE and cTACE, respectively ($P > 0.999$). Also, Sacco *et al*[31] showed statistically insignificant differences in CR and PR rates at 1 mo between DC Beads TACE and cTACE (51.5% and 48.5% *vs* 70.6% and 29.4%, respectively, $P = 0.1$). Facciorusso *et al*[32] reported single center study with early/intermediate HCC patients ($n = 249$). In this study, cTACE showed better tumor response and time to progression (TTP). OR rates were 85.3% in cTACE and 74.8% in DC Beads TACE ($P = 0.039$), and median TTP were 17 mo in cTACE and 11 mo in DC Beads TACE, respectively ($P < 0.001$).

Prajapati *et al*[33] reported the safety and efficacy of DC Beads TACE in 121 patients with advanced HCC whose median OS was 13.5 mo. DC Beads TACE was associated with a favorable prognosis, especially in those without portal vein thrombosis (PVT) and metastasis (28.9%) compared with those with PVT and metastasis (9.9%) within the Child-Pugh A group (median survival 18.8 mo *vs* 4.4 mo, $P = 0.001$). Moreover, the few minor adverse events associated with the treatment imply that DC Beads TACE is an alternative treatment strategy with advanced disease. Nevertheless, prospective studies involving more cases are needed to evaluate the efficacy and safety of TACE used drug-eluting beads in BCLC C patients.

Han *et al*[34] performed a meta-analysis of three RCTs and two case-control studies. Their meta-analysis included DC Beads TACE and cTACE groups comprising 217 and 237 patients, respectively. The results showed that DC Beads TACE tended to have better results in terms of disease control, although the difference was not significant.

CLINICAL IMPACT OF MICROSPHERES WITH SMALLER DIAMETERS

As mentioned above, several beads with smaller diameters were introduced in recent studies. Malagari *et al*[22] performed study with HepaSphere. HepaSphere 30-60 μm is a microsphere that has a dry caliber of 30-60 μm that expands to 145-213 (148 ± 45) μm after loading with doxorubicin[35]. In this report,

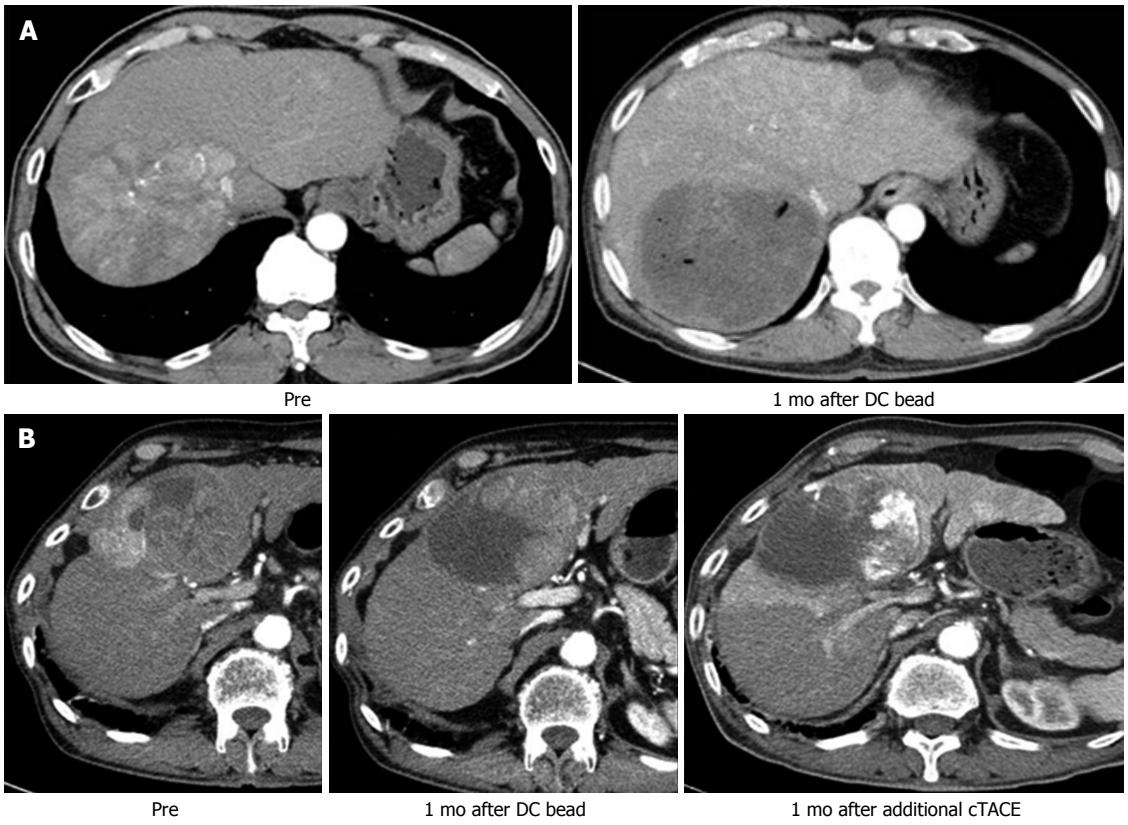

Figure 2 Difference of imaging finding between transarterial chemoembolization with drug-eluting bead and conventional transarterial chemoembolization. A: Two nodular hepatocellular carcinomas (HCCs) with arterial enhancement showed the total necrosis of HCC in follow-up liver dynamic CT after treating with DC bead; B: Nodular HCC showed nodular arterial enhancing viable portion within the partial necrosis of HCC in follow-up CT after treating with DC bead. After additional conventional transarterial chemoembolization (TACE), incomplete lipiodol uptake in remained HCC showed the matched lesion in the CT imaging. CT: Computed tomography.

HepaSphere 30-60 μm showed 68.9% of OR without serious adverse events.

Spreafico *et al*[23] performed study using TACE with M1 DC bead for HCC patients who were not indicated to resection or ablation (*n* = 45). M1 DC bead is newly developed DC bead with diameter of 70-150 μm. In this study, 77.7% of patients obtained OR (CR in 33.3%) and, 78% of tumor nodules achieved OR (CR in 42%). Thirteen patients (29%) underwent surgery after achieving successful tumor downstaging by TACE with M1. (Liver transplantation = 13, Major hepatectomy = 1). Adverse events occurred in limited cases, most of which were insignificant clinical outcomes (grade 1/2).

Malagari *et al*[24] reported study with TANDEM microsphere. TANDEM is precisely calibrated microspheres that diameters with 40, 75, and 100 μm were used in this study. Fifty-one HCC patients who were not amenable to curative therapy were enrolled in this study (mean diameter of the tumors; 7.28 ± 2.09 cm). At 6 mo follow-up, 63.82% of patients achieved OR (CR in 21.27%). And OS at 1, 2, and 3 years were 92.3%, 88.46%, 82.6%, respectively. Majority of adverse events were mild post-embolization syndrome and 4 cases of serious adverse events (grade 3-5) were reported. Patients with high loading dose of doxorubicin (150 mg) were associated with biliary damage.

EVALUATING THE TREATMENT RESPONSE TO DC BEADS TACE

To evaluate the response to cTACE, liver dynamic computed tomography (CT) should be performed 4 wk after therapy to determine the future treatment plan[36]. Especially, the treatment response to DC Beads TACE can be evaluated more definitely than can that to cTACE, in which incomplete lipiodol uptake causes difficulty with evaluating the treatment response (Figure 2)[37].

Chung *et al*[37] reported that the enhancement patterns of HCC after TACE used with beads could be useful for determining the prognosis. They analyzed images of the arterial phase of dynamic liver CT 1 mo after TACE with beads and categorized the enhancement patterns as no enhancement, peripheral ring enhancement, or peripheral nodule-like enhancement. Peripheral nodule-like enhancements suggest disease progression, while no enhancement or peripheral ring enhancements indicated a CR.

Golowa *et al*[38] showed that DEBDOX mixed with

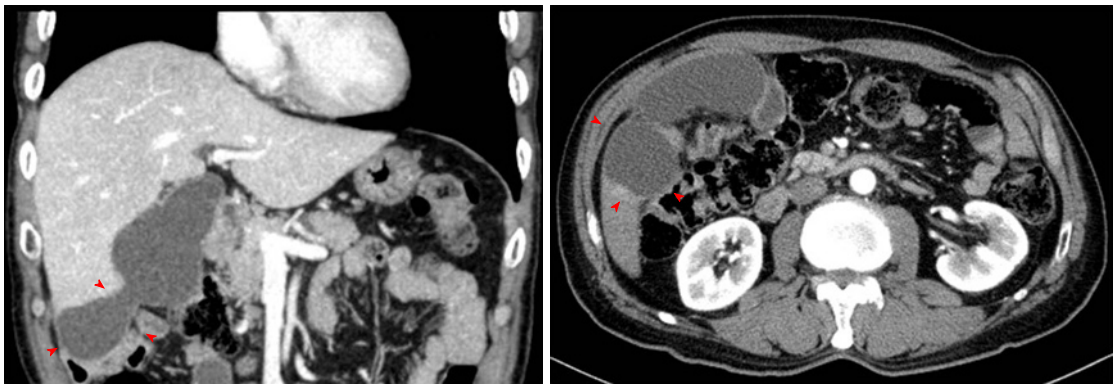

Figure 3 Biloma in a patient treated by transarterial chemoembolization with drug-eluting bead.

contrast medim helps demonstrates the precise distribution and uptake of the chemotherapeutic agent without disrupting the treatment response evaluation. The contrast medium enabled visualization of the treated tumor due to the increased attenuation, similar to that of lipiodol. Unlike lipiodol, however, the contrast medium was not retained within the tumor. Noncontrast CT immediately after TACE was helpful to evaluate the treatment response.

SURVIVAL

Malagari *et al*[39] reported a 5-year survival analysis of patients treated with DC Beads TACE. The mean OS was 43.8 (range 1.2-64.8) mo, and the OS rates at 1, 3, and 5 years were 93.6%, 62%, and 22.5%, respectively. With respect to the Child-Pugh class, the 5-years OS, rate was higher for Child class A than Child class B (29.4% *vs* 12.8%). For DC Beads TACE, the number of lesions, lesion vascularity, and local response were significant independent determinants of 5-year survival.

Burret *et al*[40] reported the survival of 104 patients with HCC treated with DC Beads TACE. The median survival was 48.6 mo. The OS rates at 1, 3, and 5 years were 89.9%, 66.3%, and 38.3%, respectively. According to the BCLC stage, the median survival and OS rates at 1, 3, and 5 years for patients with early-stage disease was 54.2 mo and 89.7%, 67.8%, 33.9%, respectively, versus 47.7 mo and 88.2%, 64.4%, and 39.4%, respectively, for patients with intermediate-stage disease. This study reported better survival rates with DC Beads than with cTACE.

Song *et al*[29] compared prognostic factors that affected survival rates in patients with HCC treated with DC Beads TACE (n = 60) *vs* cTACE (n = 69). DC Beads TACE was a significant independent factor associated with better survival (P = 0.037). Moreover, the alpha-fetoprotein level and BCLC stage were significant predictors of superior patient survival. These results suggest that the use of DEBDOX is related to a favorable outcome in patients with unresectable HCC.

However, the survival benefits of DC Beads TACE over cTACE remain controversial. Recchia *et al*[41] reported retrospective study that included 35 patients of DEB-TACE and 70 patients of cTACE. There was no statistically significant difference in median OS between cTACE and DEB-TACE (11.4 mo *vs* 18.4 mo, respectively). Facciorusso *et al*[32] also reported that median survival of early/intermediate stage HCC patients (n = 249) between cTACE and DC Beads TACE showed insignificant differences (39 mo *vs* 32 mo, respectively, P = 0.1).

In two RCTs, Golfieri *et al*[30] reported the 2 year survival rates between DC Beads TACE and cTACE (56.8% *vs* 55.4%, respectively, P = 0.949) of 117 HCC patients. Sacco *et al*[31] also showed that estimated 2-year cumulative survival rates were statistically insignificant between DC Beads TACE and cTACE (86.8% *vs* 83.6%, respectively, P = 0.96). Furthermore, Facciorusso *et al*[42] reported meta-analysis consisted of four RCTs and 8 observational studies with 1449 patients who underwent 689 DC Beads TACE and 760 cTACE. In this study, statistically insignificant trends in favor of DC Beads TACE were observed for 3-year survival rates.

SAFETY AND COMPLICATIONS

The complications reported with DC Beads include pleural effusion, gastric ulcer bleeding, cholecystitis, and abscess formation, with the prevalence ranging from 4.2% to 11.4%[10,12,16,43]. These complication rates are comparable to those of cTACE. However, it is noteworthy that no systemic complicates associated with doxorubicin have been reported in the clinical studies performed so far.

Guiu *et al*[44] reported that biloma and liver infarct were independently related to DEB-TACE (OR = 9.78, P = 0.002) (Figure 3). Interestingly, a non-cirrhotic underlying liver was a strong independent risk factor for developing biloma and liver infarct (OR = 8.125, P = 0.04). The role of a hypertrophied peribiliary plexus as a porto-arterial shunt may account for the association observed in this study[45]. These changes seen in the cirrhotic liver reinforce collateral formation,

which prevents further ischemic and chemical injury in the bile ducts.

Systemic complications related to doxorubicin include neurological injury, pulmonary edema, bone marrow suppression, and gastrointestinal problem (nausea and vomiting). Especially, dose-dependent cardiomyopathy (adriamycin-induced congestive heart failure) limits its long-term use. Advanced age and left ventricular dysfunction increase the risk of this complication. At a cumulative doxorubicin dose exceeding 450 mg/m^2, it is important to monitor for this high-risk of cardiovascular complication[46]. It is worth noting that no doxorubicin-related systemic complications have been observed in clinical studies of DC Beads TACE performed to date.

ASSESSMENT OF COST EFFECTIVENESS

Cucchetti *et al*[47] investigated the cost effectiveness of DC Beads TACE. In meta-analysis, patients with cTACE experienced significantly frequent post-TACE syndrome (P = 0.018) and longer hospitalization (P = 0.01). DC Beads TACE earned 4.0 quality-adjusted life-years (QALYs) while cTACE earned 3.3 QALYs. Total costs of DC Beads TACE were € 11656 and those of cTACE were € 10389. DC Beads TACE spent higher costs than cTACE, but, higher QALYs were achieved from the treatment. Expected cost-effectiveness for DC Beads TACE was € 3089/QALY and that of cTACE was € 3246/QALY. Improvement of quality of life could be attained by DC Beads TACE with modest increment of costs.

CONCLUSION

Intermediate- and advanced-stage HCCs remain a challenging to physicians and interventional radiologists because of suboptimal tumor control and frequent relapse even after inducing complete tumor necrosis using a catheter-based approach. The potent anticancer effect of drug-eluting beads administered through the hepatic arteries might complement standard therapeutic modalities[34,48]. DC Beads are relatively new embolic agents that allow sustained release of chemotherapeutic agents and minimize systemic side effects. The advantages of DC Beads TACE are better synergistic effects with embolization and cytotoxic effects and minimized system toxicity compared with cTACE. Especially, regarding the treatment results for unresectable single tumors or multiple tumors (intermediate stage), Child B class, and recurrent HCC, TACE used with drug-eluting Beads resulted in a better treatment response rate than did cTACE. However, cTACE may provide better outcomes than those of DEBDOX TACE, when superselective embolization of the small feeding vessels is possible.

The biliary damage caused by DC Beads TACE should be weighed against their use in selected patients. A more careful therapeutic approach to prevent of biloma or liver infarct development is needed in patients with an underlying non-cirrhotic liver. For effective TACE with DEBDOX, the duration, distribution, and dosage of the drug delivered to the tumor and surrounding non-tumor tissue are most important[19]. We must consider the doxorubicin dose, diameter of the DC Beads and tumor vascularity required for the optimal treatment response before performing TACE with DEBDOX.

DC Beads TACE may provide improved survival rates and quality of life to some extent as compared to cTACE[32,41,47,49]. However, several RCTs and meta-analysis do not demonstrate significant survival advantages of DC Beads TACE[30,31,42]. Because of relatively small numbers of prospective randomized trials, further investigations with well designed, large scaled, comparative studies searching for the long-term survival are necessary. DC Beads TACE seemed to afford better cost-effectiveness than cTACE[47]. However, previous studies were based on various conditions involving different countries and institutes in terms of costs, clinical circumstances, and technical procedures. Study with standardized technical protocols and performances will be needed to validate the cost-effectiveness of DC Beads TACE.

Indeed, it remains inconclusive as to the superiority of DC Beads TACE over cTACE. Decision to performing DC Beads TACE or cTACE needs to be tailored in each individual patient depending on his or her economic status, physician's experiences, and expertise of institute. Future studies are warranted to determine the appropriate indications of DC Beads TACE.

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P- Reviewer: Hoyos S, Mazzaferro V **S- Editor**: Qi Y **L- Editor**: A **E- Editor**: Zhang FF

World Journal of Gastroenterology

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Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.3748/wjg.v22.i40.8862

World J Gastroenterol 2016 October 28; 22(40): 8862-8868
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

Liver-targeted hydrodynamic gene therapy: Recent advances in the technique

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Supported by in part Grant-in-Aid for Scientific Research from the Japanese Society for the Promotion of Sciences, No. 26860354 to Kamimura K, No. 16K19333 to Yokoo T, and No. 26293175 to Terai S.

Conflict-of-interest statement: The authors declare that they have no current financial arrangement or affiliation with any organization that may have a direct influence on their work.

Manuscript source: Invited manuscript

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Received: July 4, 2016
Peer-review started: July 6, 2016
First decision: July 29, 2016
Revised: August 3, 2016
Accepted: August 23, 2016
Article in press: August 23, 2016
Published online: October 28, 2016

Abstract

One of the major research focuses in the field of gene therapy is the development of clinically applicable, safe, and effective gene-delivery methods. Since the first case of human gene therapy was performed in 1990, a number of gene-delivery methods have been developed, evaluated for efficacy and safety, and modified for human application. To date, viral-vector-mediated deliveries have shown effective therapeutic results. However, the risk of lethal immune response and carcinogenesis have been reported, and it is still controversial to be applied as a standard therapeutic option. On the other hand, delivery methods for non-viral vector systems have been developed, extensively studied, and utilized in *in vivo* gene-transfer studies. Compared to viral-vector mediated gene transfer, nonviral systems have less risk of biological reactions. However, the lower gene-transfer efficiency was a critical hurdle for applying them to human gene therapy. Among a number of nonviral vector systems, our studies focus on hydrodynamic gene delivery to utilize physical force to deliver naked DNA into the cells in the living animals. This method achieves a high gene-transfer level by DNA solution injections into the tail vein of rodents, especially in the liver. With the development of genome editing methods, *in vivo* gene-transfer therapy using this method is currently the focus in this research field. This review explains the method principle, efficiency, safety, and procedural modifications to achieve a high level of reproducibility in large-animal models.

Key words: Gene therapy; Liver; Hydrodynamic gene delivery; Non-viral; Image-guided

Core tip: Among a number of nonviral vector systems, hydrodynamic gene delivery has been used to study human diseases. The major advantage of the method is the simple and easy step to deliver naked DNA into living animal cells by physical force. The original method modification of injecting the DNA solution into a rodent tail vein has made it applicable in large animals. This method of delivering naked DNA can contribute to treat, not only liver disease but also other systemic diseases that can be cured by facilitating/altering gene expression through the liver.

Yokoo T, Kamimura K, Abe H, Kobayashi Y, Kanefuji T, Ogawa K, Goto R, Oda M, Suda T, Terai S. Liver-targeted hydrodynamic gene therapy: Recent advances in the technique. *World J Gastroenterol* 2016; 22(40): 8862-8868 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i40/8862.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i40.8862

INTRODUCTION

The liver is the largest organ in the body and is the center of numerous metabolic pathways. Therefore, it is involved in various inherited diseases. These diseases are often caused by a critical gene-product deficiency or overproduction in the hepatocytes. Recent advances in diagnostic strategies using molecular biology and genetics have helped establish various therapeutic methods. Moreover, genetic abnormalities are altered by introducing a gene-coding sequence or a nucleic-acid sequence into these cells to inhibit the specific gene overexpression. Thus, liver-targeted gene therapy emerged as a promising therapeutic strategy. Originally, human gene therapy was first performed using a retrovirus-mediated *ex vivo* gene-delivery method to target adenosine-deaminase deficiency in 1990[1]. However, serious adverse events occurred in the following years, including lethal immune reaction to the adenovirus vector and oncogenesis because of genetic transformation caused by the retrovirus vector[2-4]. Over this time period, viral vectors have been improved toward higher levels of safety and efficiency, yet concerns regarding biological safety, including lethal immune reaction and oncogenesis, have remained. On the other hand, various nonviral gene-delivery methods have also been extensively studied for use in clinical applications. However, the major obstacle of the methods was lower gene-delivery efficiency compared to the viral vectors. Many ongoing studies modify the current strategies to provide a better gene-delivery efficiency while maintaining its safety features. Among these methods, this report focuses on the hydrodynamic gene-delivery (HGD) method for human gene delivery. The liver-targeted HGD efficacy and safety are described, including recent progress of the procedure applied toward clinical application. We hope the information described will help physicians to understand the principles of HGD and lead to new strategies to better treat patient diseases compared to the conventional treatment methods.

NUCLEIC-ACID DELIVERY TO THE LIVER AND THE PRINCIPLE OF HGD

Concerns regarding carcinogenesis and immune reaction because of viral-vector-based gene transfer have inspired the efforts to develop methods of nucleic-acid delivery in its naked form *in vivo*. The objective of naked nucleic-acid delivery is either: (1) express gene of which the product is missing or low level; (2) gene vaccination; (3) inhibition of specific gene expression; and (4) deliver necessary parts of genome editing; *etc*. Table 1 summarizes the nonviral methods of nucleic-acid delivery for liver-target gene delivery. The barriers for nucleic-acid delivery to hepatocytes are the plasma membrane and the endothelium in the sinusoidal structure. Therefore, the physical methods of intrahepatic nucleic-acid delivery included needle injection, gene gun, electroporation, sonoporation, and HGD. They were developed to overcome the structural barriers using physical forces of pressure, shock wave, electric pulse, ultrasound wave, and hydrodynamic pressure. Among these methods, HGD to the mouse model was reported as an easy and effective *in vivo* gene-delivery method by injecting naked DNA solution into the tail vein[5,6]. Various genes were delivered into rodent hepatocytes to analyze their function and to examine the therapeutic effect within the research fields of gastroenterology and hepatology[5,7-15] (Table 2).

The principle of HGD relies on the mechanical force developed using a quick injection of a large amount of plasmid volume. Briefly, a 10% body weight DNA solution is injected within 5-7 s into a 20 g mouse. This force created a transient congestion in their right ventricle allowing the solution to flow back into the hepatic veins. Next, the solution passes through the sinusoidal structure to the portal veins allowing the force to make transient pores in the hepatocyte cell membrane[16-19]. Then, nucleic acids enter into the hepatocytes, move to the nucleus, and finally facilitate targeted gene expression. The transient pores naturally disappears in a short period[18], and the exogenous gene can be expressed in the hepatocytes. Due to the large amount of solution and its rapid flow rate, the blood is transiently cleared away from the vessel, and there is no concern regarding DNA degradation in the blood by DNase. A number of transfected cells are confirmed in the overall targeted area in the liver, although relatively higher gene-delivery efficiency was seen in the local areas highly impacted by the injection flow. The major advantage of the method is a less risk

Table 1 Non-viral gene delivery systems toward the liver

Method	Functional component
Lipids	Cationic lipids
Polymers	Cationic polymers
Proteins	Natural or chemically modified proteins in cationic nature
Peptides	Lysine or arginine residues in peptides
Needle injection	Mechanic force
Gene gun	Pressure
Electroporation	Electric pulse
Sonoporation	Ultrasound
Hydrodynamic delivery	Hydrodynamic pressure

Table 2 Summary of the applications of hydrodynamic delivery for functional analysis of therapeutic genes related to the diseases of gastroenterology and hepatology

Disease	Therapeutic Genes	Ref.
Nonalcoholic steatohepatitis	Inducible nitric oxcide synthetase	[8]
Hepatitis	HBV knockdown HCV knockdown	[5,9]
Fulminant hepatitis	NKG2D knockdown osteopontin knockdown	[7,10]
Liver injury	c-met IL-37 caspase knockdown	[7,11]
Liver fibrosis	platelet-derived growth factor receptor beta knockdown	[12]
Liver Regeneration	fibroblast growth factor 7	[13]
Fabry disease	alpha-galactosidase A	[7]
Pancreatitis	pancreatitis associated protein 1	[14]
Colon cancer	IL-15	[15]

HBV: Hepatitis B virus; HCV: Hepatitis C virus; IL: Interleukin.

of immune response and oncogenesis. Specifically, naked DNA plasmids and saline do not possess any immunogenicity reagents or the potential of DNA integration, compared to the chemical compounds used for viral or other nonviral gene-delivery methods.

EFFICIENCY AND SAFETY

The efficiency of the original procedure was confirmed, and various genes were examined for therapeutic effects in mouse disease models[5,7-15]. Only one procedure transfected a large number of hepatocytes with a specific protein secreting gene, approximately 40% of hepatocytes in a targeted area, leading to a high level of gene expression in the liver and body[5]. In 2013, we reported that only one delivery method could achieve a high level of FIX expression in rats, which was expected to stop bleeding in patients with hemophilia B[20]. Regarding its safety, the impact of hydrodynamic injection to the liver is known to only elicit slight liver damage. In a microscopic study, destruction of cells and tissue was merely seen in the liver[5,18]. Also, transient abnormal aminotransferase (ALT) increases recover in a short term, and there are no signs of hepatic failure[20,21]. Considering the half-life of ALT and the small number of destroyed hepatocytes, we hypothesize that the ALT increase is derived from leakage out of the newly created transient pores and not from destroyed hepatocytes.

Based on this method's efficiency and safety shown in rodent studies, recent efforts have safely applied this method for large-animal studies to show the clinical applicability. The essential key was to decrease the injection volume of 10% BW used in rodents, which is 5 L in 50-kg patient, to maintain the gene-delivery efficiency. Several reports showed the modification of the original procedures for this purpose, and we have applied catheter-based, target-organ-specific, and target-site-specific HGD in 2009[22]. By this procedure, the hepatocytes were hydrodynamically delivered genes of interest with < 1% of BW solution in each liver lobe. As a result, the gene-delivery efficiency was maintained showing a therapeutic level of human factor IX expression in dogs (manuscript in preparation). This procedure involves a catheter insertion through the jugular vein to each hepatic lobular vein. This is followed by the hydrodynamic injection of naked DNA plasmid solution with temporally occlusion of blood flow using balloon placed at the tip of the catheter (Figure 1A). By this technique, a sufficient intravascular pressure was provided upon the hydrodynamic injection (Figure 1B), a key of successful gene transfer. In addition, no significant solution leakage with the added pressure is seen within the systemic dynamics, which normally impacts cardiopulmonary function. The safety and impact of HGD in large-animal models were carefully evaluated in previous reports as well[22-24]. Hydrodynamics of the procedure were also validated using CT scans during the injection[25]. These studies confirmed the site specificity of the gene-delivery efficiency, and the target-region-specific impacts caused by the injection. In addition, histochemical analyses of the transiently expanded sinusoidal structure showed the same as the phenomena seen in small animals and recovered within a few days[24]. While the systemic inflammatory cytokines including interferon-α, interleukin (IL)-6, IL-8, IL-18, and IL-4 showed increase in mice after HGD through their tail vein affecting the systemic condition, however, the liver-targeted HGD showed an increase in cytokines related to the myocytes and vascular stretching including tumor necrosis factor-α, IL-10, MCP-1, and Canine KC, but not in systemic inflammatory cytokines[24]. This is probably due to the localized effect of injection pressure, flow, *etc*. Recently, laparoscopy was used to monitor the change in the lobe of the liver upon injection to confirm the site specificity and overall impact on the lobe (Figure 1C). The findings presented the precise site-specific distribution of the DNA solution upon the liver-targeted, lobe-specific HGD resulted in the site-specific expression of injected transgene (Figure 1D). Using a computer-controlled injection device (Figure 2), HGD was performed to the right lateral lobe with approximately 1.5% BW

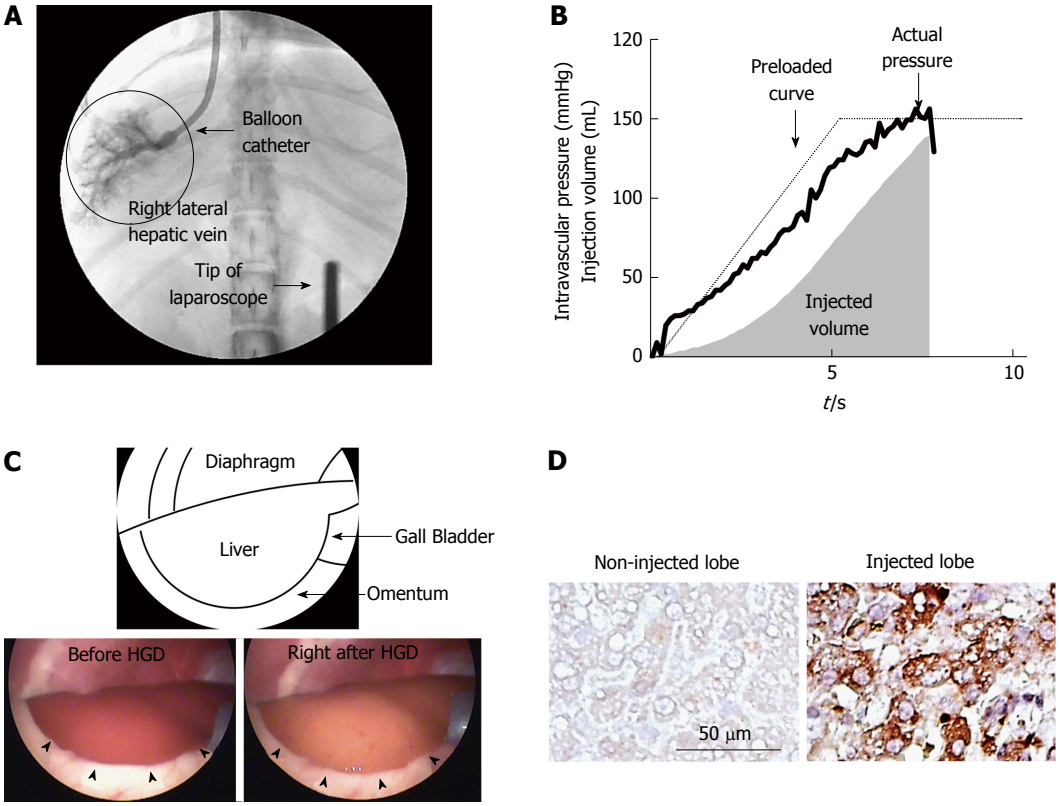

Figure 1 Image-guided, computer-controlled hydrodynamic gene-delivery to the dog liver. The balloon catheter was placed at the appropriate position in the hepatic veins of right lateral lobe and the occlusion of the blood flow by the balloon was confirmed by injecting a small amount of contrast medium into the hepatic vein. Then the hydrodynamic injection of naked DNA solution was performed under the real time monitoring of liver structure by the laparoscope using the computer-controlled injection system (A). B: Time-pressure curve and the volume of injected solution recorded in the injection system. Solid and dotted lines represent actual and preloaded time-pressure curves. A gray area shows cumulative volume of injected saline (mL). C: Laparoscopic findings of the hydrodynamically injected right lateral lobe of the dog. The injected lobe was swollen and the injected DNA solution transiently made the liver pale. No destruction nor bleeding were seen on the surface of the liver (arrowheads). D: The effect of lobe-specific hydrodynamic gene delivery of luciferase expressing plasmid. The immunohistochemical analyses showed positively stained cells in the injected right lateral lobe. No stained cells were found in non-injected left lateral lobe.

solution completed within 7.5 s (Figure 1). Although the hydrodynamically injected DNA solution transiently made the liver pale, no destruction nor bleeding were seen.

INNOVATION OF INJECTION SYSTEM

Overall, the catheter-based procedure of liver-targeted, lobe-specific HGD showed safe and efficient gene delivery in large-animal models. Therefore, the next step toward clinical application was to ensure the procedure reproducibility using various gene targets. For the liver, the size and elasticity of its tissue can vary between species, age ranges, and even individual subjects. In addition, it is known that many liver diseases show liver fibrosis during their final stage. During HGD, the specific intravascular pressure resulted in various patterns and differences in the gene-delivery efficiency with the same injection pressure (*i.e.*, hydrodynamic pressure). Therefore, the intravascular pressure was used to control the injection speed, and the efforts have been made to develop the computer-controlled HGD system[20,21]. The system was set to control the injection power to reproduce the intravascular pressure during HGD to any target. Specifically, the efficient and safe HGD to the liver is associated with a peak pressure level in the vessel and tissue, duration of the pressure, and concentration of plasmid solution (Figure 2). The reproducibility in gene-delivery efficiency for any target is the advantage of using this system. The system function involves: (1) preload of an arbitrary time-pressure curve to the computer; (2) placement of a sensor to detect an intravascular pressure at the portal vein; (3) insertion of a catheter for a hydrodynamic injection into the inferior vena cava (IVC); (4) beginning of a hydrodynamic injection after occluding the supra- and infra-portions of the hepatic IVC; (5) transmission of the intravascular pressure data to the computer every 50 milliseconds; (6) regulation of the injector power to reproduce the time-pressure curve, which was input before the injection; and (7) repetition of step 5 and 6 until injection completion (Figure 2). Further, the flexibility of the system can control various types of time-pressure curves *in vivo*[20] so that the safe, efficient, and reproducible injection can be repeatedly performed. Overall, this computer-controlled injection system has the potential of achieving reproducible HGD, even if anyone performs the procedure for any kind of target (Figure 2)[21-24].

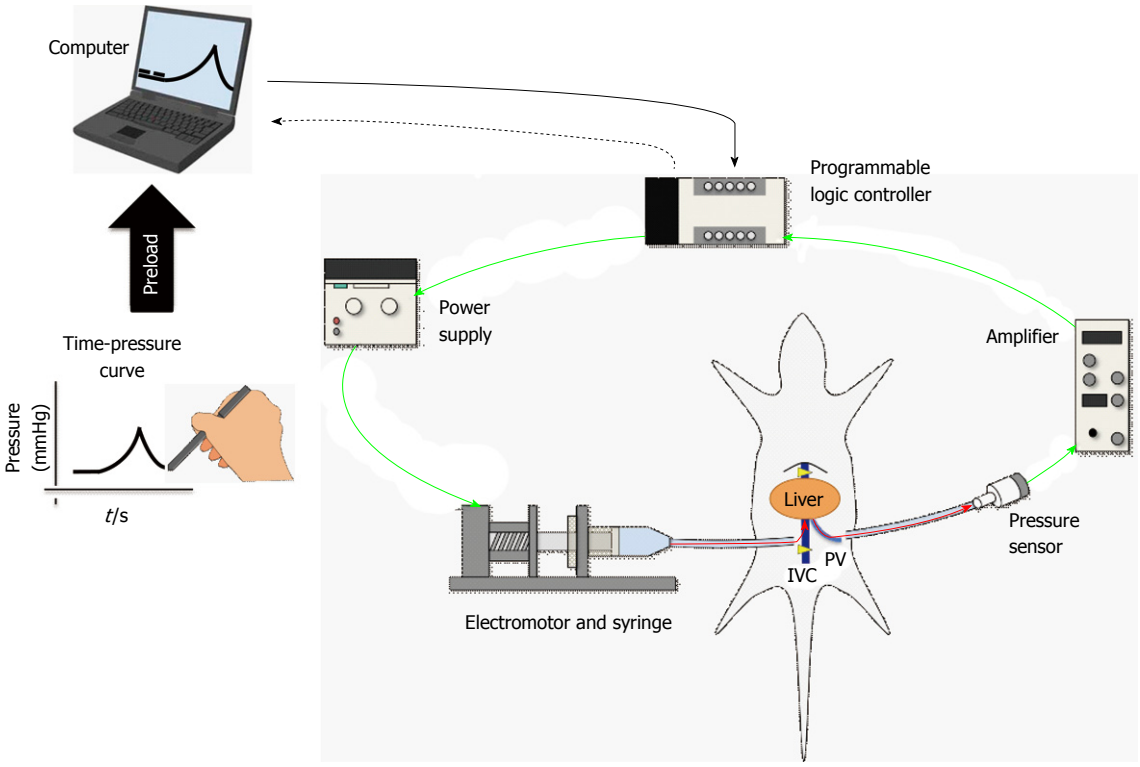

Figure 2 Scheme of computer-controlled injection system. The schema of the newly developed hydrodynamic gene-delivery system. This figure is partly reused and modified with updated information from Figure 1 in Ref [20] with their permission. IVC: Inferior vena cava; PV: Portal vein.

Our recent and current studies[26,27] utilizing real-time monitoring of the computer-controlled injection under fluoroscopy and laparoscopy (Figure 1) demonstrated its precise controllability. Therefore, the current ongoing studies are focusing on treating diseased-animal models, such as liver fibrosis with the different liver structure characteristics and various levels of fibrosis. This scenario provides the best disease model to examine the intravascular-pressure-based injection control. For these purposes, we are continuously modifying the injection system for its clinical trial application, in collaboration with GMP-grade engineers, physicians, and industrial companies.

GENE THERAPY THROUGH THE LIVER

While improving the injection system, we are investigating hepatic gene therapy for systemic diseases as well as for the liver diseases. The target diseases include hemophilia, human alpha-1 antitrypsin deficiency, *etc.* which can be treated by increasing the hepatic expression of normal proteins in the liver and then their secretion into blood plasma. For liver disease gene therapy, Abe *et al*[28] recently reported that HGD-mediated MMP13 expression in the rat liver prevented liver fibrosis. Surprisingly, rat liver treated by *MMP13* gene therapy did not suffer from significant fibrosis after at least 10 wk. Hyaluronic acid levels of MMP13-treated rats with bile-duct ligation were statistically equivalent to those of normal rats. Therefore, MMP13 is a promising candidate for liver fibrosis gene therapy. Further studies focusing on the therapeutic effect in advanced stage liver fibrosis are currently ongoing. In addition, HGD procedure from hepatic artery is also being examined in our lab to treat hepatocellular carcinoma by this method since hepatocellular carcinoma is fed by the hepatic artery. While early study showed transient increase of platelet count injecting large volume of thrombopoietin-expressing plasmids into human hepatic veins[29], which is the only human trial to date, strict adjustment of injection parameters and setting of the system are necessary to apply HGD for human.

CONCLUSION

Nucleic-acid-based medicine is quickly developing with the detailed analyses of disease-related genes. A simple, safe, effective, and reproducible method is essential before applying this strategy to human diseases. The development of HGD-based gene therapy for large animals provides a great milestone to this point, and further studies are necessary to make the procedure clinically applicable.

ACKNOWLEDGMENTS

The authors would like to thank all members at the Niigata city industrial promotion center and for their excellent assistance in producing the system. The authors would also like to thank Yoshihiko Ohba for the supporting of fine-tuning of the system. They also

thank Enago for the critical reading of the manuscript and English language review.

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P- Reviewer: Alino PelliIcer SF, Liu HY, Sanal MG, Strom SC, Tomizawa M
S- Editor: Gong ZM **L- Editor**: A **E- Editor**: Zhang FF

World Journal of Gastroenterology

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Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.3748/wjg.v22.i40.8869

World J Gastroenterol 2016 October 28; 22(40): 8869-8882
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

REVIEW

Cardiovascular risk after orthotopic liver transplantation, a review of the literature and preliminary results of a prospective study

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Author contributions: All authors contributed to this paper; Pisano G, Fracanzani AL and Fargion S contributed to study concept, literature review and analysis, drafting of the manuscript and critical revision; Caccamo L and Donato MF contributed to acquisition and analysis of patients data; all authors approved the final version.

Conflict-of-interest statement: Authors declare no conflict of interests for this article.

Manuscript source: Invited manuscript

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Received: June 29, 2016
Peer-review started: June 30, 2016
First decision: August 8, 2016
Revised: August 27, 2016
Accepted: September 28, 2016
Article in press: September 28, 2016
Published online: October 28, 2016

Abstract

Improved surgical techniques and greater efficacy of new anti-rejection drugs have significantly improved the survival of patients undergoing orthotopic liver transplantation (OLT). This has led to an increased incidence of metabolic disorders as well as cardiovascular and cerebrovascular diseases as causes of morbidity and mortality in OLT patients. In the last decade, several studies have examined which predisposing factors lead to increased cardiovascular risk (*i.e.*, age, ethnicity, diabetes, NASH, atrial fibrillation, and some echocardiographic parameters) as well as which factors after OLT (*i.e.*, weight gain, metabolic syndrome, immunosuppressive therapy, and renal failure) are linked to increased cardiovascular mortality. However, currently, there are no available data that evaluate the development of atherosclerotic damage after OLT. The awareness of high cardiovascular risk after OLT has not only lead to the definition of new but generally not accepted screening of high risk patients before transplantation, but also to the need for careful patient follow up and treatment to control metabolic and cardiovascular pathologies after transplant. Prospective studies are needed to better define the predisposing factors for recurrence and *de novo* occurrence of metabolic alterations responsible for cardiovascular damage after OLT. Moreover, such studies will help to identify the timing of disease progression and damage,

which in turn may help to prevent morbidity and mortality for cardiovascular diseases. Our preliminary results show early occurrence of atherosclerotic damage, which is already present a few weeks following OLT, suggesting that specific, patient-tailored therapies should be started immediately post OLT.

Key words: Orthotopic liver transplant; Cardiovascular risk; Atherosclerosis, Non-alcoholic fatty liver disease; Intima-media thickness; Epicardial fat thickness; Diastolic dysfunction

Core tip: Due to better immunosuppressive therapies, the survival of liver transplantation recipients is improved, but an increased incidence of metabolic disorders as well as cardiovascular and cerebrovascular diseases as causes of morbidity and mortality is observed. This review analyzes risk factors [before orthotopic liver transplantation (OLT) and occurring *de novo* after OLT] leading to cardiovascular diseases and the current tools to identify high risk patients. We also provide preliminary data from one of the first prospective studies on the evolution of cardiovascular damage in adult patients submitted to OLT.

Pisano G, Fracanzani AL, Caccamo L, Donato MF, Fargion S. Cardiovascular risk after orthotopic liver transplantation, a review of the literature and preliminary results of a prospective study. *World J Gastroenterol* 2016; 22(40): 8869-8882 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i40/8869.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i40.8869

EVIDENCE OF CARDIOVASCULAR DISEASE IN PATIENTS SUBMITTED TO ORTHOTOPIC LIVER TRANSPLANTATION

Orthotopic liver transplantation (OLT) represents the only therapy for several end stage liver diseases of different etiology. In Europe as well as in United States nearly 6000 patients/year are submitted to liver transplantation[1,2].

According to the United Network for Organ Sharing registry, the survival rate at 1, 5 and 10 years after OLT is respectively of 85%, 70% and 50%[2]. Similarly, according to the European Liver Transplant Registry[1], the survival rate at 1, 5, 10, 15, 20 years after OLT is of 82%, 71%, 61%, 51% and 43%. However, various diseases have emerged as possible causes of post OLT complications. During the first 6 mo post-transplant, the highest risk of death was observed (11% mortality rate), while between 6 mo and 8 years post OLT, it rated at 2.5%-5%. Such mortality value increased again after 8 years to 6%-7%.

In a recent study including 798 transplanted subjects followed for a median of 10 years[3], in which 327 deaths were reported, malignity was the first cause of death followed by cardiovascular causes, infective diseases and renal failure, accounting for 22%, 11%, 9% and 6% of death, respectively.

As cardiovascular diseases emerged as a leading cause of death, several studies have been proposed in order to understand the predisposing factors leading to cardiovascular disease, as well as the post-OLT conditions facilitating cardiovascular morbidity (*de novo* diseases). However, scanty data are available in prospective studies.

RISK FACTORS OF CARDIOVASCULAR COMPLICATIONS AFTER OLT

Pre-existing cardio-metabolic pathologies and *de novo* occurrence, partly associated with immunosuppressive therapy, are considered the main causes of post-transplant cardiovascular complications[4].

Pre-existing metabolic factors/non-alcoholic fatty liver disease

The relevance of cardiometabolic pathologies reflects the ongoing epidemic of obesity and diabetes in the United States[5,6] and all over the Western countries. This is further documented by the marked increase in the prevalence of diabetes among candidates to OLT independently of etiology. Another metabolic disease recently recognized to have a strong role in OLT is non-alcoholic fatty liver disease (NAFLD), considered a manifestation of the metabolic syndrome (MS) or even suggested to precede MS, of which insulin resistance is the hallmark. NAFLD is the most frequent cause of liver disease in Western countries and is likely to become the most common indication for OLT over the next decade[7,8].

Non-alcoholic steatohepatitis (NASH) represents 20%-25% of all NAFLD and may potentially evolve to cirrhosis and hepatocellular carcinoma, besides carrying all cardiovascular risks typically associated to MS[9]. Interestingly, Targher *et al*[10] reported that NASH-affected patients are at increased risk of atrial fibrillation, which was recently identified as a severe risk factor for OLT[11]. In an analysis performed by Van Wagner *et al*[12], atrial fibrillation was one of the factors independently associated with major adverse cardiovascular events, especially in patients with a previous history of NASH and alcoholic cirrhosis. Indeed, a positive history of atrial fibrillation before liver transplantation was significantly more frequent in patients with major adverse cardiovascular events than in those without a previous episode of atrial fibrillation[13].

Several studies pointed out the relationship between NAFLD and cardiovascular mortality in patients submitted to OLT. Overweight/obesity, dyslipidemia, hypertension and glucose metabolism abnormalities

are typical alterations detected with a high frequency in patients with NAFLD and they also define MS., notably, they have all been associated with high morbidity and mortality in patients submitted to OLT[14,15]. In addition, patients with NAFLD have been reported to be at risk for chronic kidney disease, which is another known risk factor for CVD[16,17]. The strong association between NAFLD and chronic kidney disease suggests that NAFLD could be used to stratify patients undergoing liver or kidney transplantation for a better evaluation of CV risk[18].

In line with the importance of NAFLD in the history of patients undergoing OLT, Laish *et al*[19] found in a retrospective analysis that pretransplant NAFLD, body mass index, diabetes, and triglycerides levels were predisposing factors for the recurrence of post-transplant MS and that post-transplant MS was associated with cardiovascular morbidity and mortality.

Alteration of glucose metabolism, one of the major complications of NAFLD/MS, is already recognized to be associated with a worse prognosis (increased risk of cirrhosis and hepatocellular carcinoma occurrence) in patients with chronic liver disease, independently from the etiology (HCV chronic hepatitis, NAFLD). In particular, its presence is also associated with a worse clinical history of transplanted patients. Several studies performed in large cohorts of transplanted patients reported that both patients and graft survival was negatively associated with pre-existing diabetes or with *de novo* occurrence of diabetes after OLT[20,21].

In addition, it has been reported that subjects with type 1 diabetes had a significantly lower survival than subjects with type 2 diabetes and, in turn the latters had a reduced survival compared to patients without diabetes[20].

The role of diabetes and NAFLD in the natural history of OLT is further demonstrated by the evidence that patients undergoing OLT for NASH related cirrhosis showed significantly higher risk of CVD, either in the first 30 d[22] or within 3 years post OLT, compared with patients undergoing transplantation for other chronic liver diseases such as primary biliary and sclerosing cholangitis[23]. However, overall survival did not differ. In addition, a strong association between adverse CVD events and post-transplant hypertension and diabetes was observed, with a double risk of CVD if both comorbidities coexisted. However, conflicting data were recently obtained in a large prospective study performed in United Kingdom, including almost 4000 subjects recipients of liver transplant, with diabetes having no impact on mortality at any time after OLT[24-29]. The Authors have speculated that the intensive screenings for cardiovascular complications in the diabetic liver transplant candidates of the cohort studied could explain these unexpected results. Thus, they emphasize the importance of a careful screening and selection of the candidates to OLT, of their follow up as well as of an active diabetes management after transplantation. Such procedures could lead recipients with diabetes to have outcomes comparable to those of recipients without diabetes.

Although malnutrition is commonly observed in patients with end stage liver disease, obesity is a metabolic problem that impacts negatively both on immediate and long-term survival. Most patients in the United States who underwent liver transplantations between 1988 and 1996 were overweight, reflecting the epidemic of obesity. Obesity was more common in women and in patients with cryptogenic cirrhosis, suggesting that the so called cryptogenic cirrhosis was in truth a metabolic cirrhosis. Severe obesity (BMI > 40 kg/m^2) was associated with decreased 30-d, 1-year, and 2-year survival, while five-year survival was reduced even in patients with BMI > 35 kg/m^2[30].

DE NOVO OCCURRENCE OF METABOLIC ALTERATIONS AFTER OLT

It is very well known that recurrence or *de novo* occurrence of NAFLD post OLT as well as after kidney transplantation may facilitate MS happening[21,31-37]. However it is difficult to define the prevalence of MS after OLT due to malnutrition, which is usually present in most cirrhotic candidates to OLT, and which ameliorates after transplantation. This is followed, in the first years after OLT, by a marked increase in body weight, which in almost 20% of cases reaches the level of obesity with an increase of BMI of 60%-70% compared to the pretransplant one. The increase in weight is almost always associated with the development of NAFLD, which in turn is accompanied by insulin resistance, the hallmark of MS and of NAFLD, present in 20%-58% of cases, glucose intolerance/diabetes, altered lipids metabolism in 50%-70%, and very often hypertension in 60%-70%[21,36,38-41]. Altogether, this also contributes to the induction of systemic and renal vasoconstrition, as well as impaired sodium excretion when treated with immunosuppressive drugs.

Interestingly, obesity in liver donors is also a predictor of liver steatosis in the liver recipients, and the presence of steatosis in the donor liver is strongly related to decreased allograft function and patient survival, with a high probability of NASH development. This has led to the decision that grafts with steatosis greater than 60% cannot be used for liver transplant[22].

New-onset diabetes mellitus, a well described complication following solid organ transplantation (liver, lung and kidney), occurs in 2% to 53% of all solid organ transplants, in 4% to 25% of renal transplant recipients and in 2.5% to 25% of liver-transplants[42-48] and is associated with an increased risk of cardiovascular morbidity and infection, as well as reduced quality of life, impaired graft function and lower patient survival[21,43,48,49]. *De novo* diabetes after OLT has been associated with hepatitis C virus

Table 1 Incidence or prevalence of risk factors of the different manifestations of the metabolic syndrome after orthotopic liver transplantation

Disease	Incidence/prevalence	Risk factors	Ref.
Diabetes mellitus	9%-21% (incidence)	Male gender High pre-LT BMI Family history Hepatitis C Older age immunosuppressants rapamycin gene polymorphisms TCF7L2 gene polymorphisms (donor)	[65,105-107]
Hyperlipidemia	45%-69% (prevalence)	Diet Older age High BMI DM Renal impairment, immunosuppressants low-density lipoprotein receptor gene polymorphism (donor)	[38,108-110]
Arterial hypertension	60%-70% (prevalence)	Obesity Older age Impaired glycemia Immunosuppressants	[106,111,112]
Overweight-obesity	24%-31% (prevalence)	High BMI before LT Diet Immunosuppressants	[113-116]
Metabolic syndrome	40%-60% (prevalence)	Older age Obesity and increased BMI pre-LT DM Genetic polymorphisms in the living donor High-dosage immunosuppressive drugs Changes in intestinal microbiota	[33,106,117,118]
NAFLD/NASH	18%-100% (incidence of NAFLD in NASH and cryptogenic recipients) 0%-14% (incidence of NASH in NASH and cryptogenic recipients) 10%-40% (incidence of NAFLD in non-NASH or cyptogenic recipients)	DM Obesity and weight gain, dyslipidemia Genetic predisposition (presence of the rs738409-G allele of the Patatin-like phospholipase) Arterial hypertension Immunosuppressant pre-LT alcoholic cirrhosis Liver graft steatosis	[18,33,80,119-124]

NAFLD: Non-alcoholic fatty liver disease; NASH: Non-alcoholic steatohepatitis.

infection, pre-existing NAFLD, increased BMI, use of tacrolimus (as opposed to ciclosporine), steroids, age, and ethnicity[43,44,50-52]. In kidney transplant recipients variables predictors of new onset diabetes were similar to those of OLT, being crucial the early detection and management. Table 1 lists risk factors leading to metabolic syndrome and its different clinical manifestations after OLT.

IMMUNOSUPPRESSIVE THERAPY AND METABOLIC ALTERATIONS

The use of highly effective anti-rejection medications has led to improved survival, albeit with evidence of well-recognized side effects such as metabolic derangements, and an overall increase in MS and insulin resistance after OLT.

Steroids decrease insulin production by beta-cells, increase gluconeogenesis and reduce glucose utilization, thus strongly contributing to the occurrence of diabetes and weight gain. Tacrolimus and cyclosporine A, the calcineurin inhibitors (CNIs), facilitate *de novo* occurrence of diabetes by decreasing insulin production and inducing insulin resistance, which is followed by hyperinsulinemia, the effect being more severe for tacrolimus. In addition to these effects increased oxidative stress and lipid peroxidation also occur, followed by hypertension, dyslipidemia and kidney damage[36,53-64].

Among other immunosuppressive drugs demonstrated to exert negative cardiovascular effects is sirolimus, which was reported to be complicated by serious adverse events including hepatic artery thrombosis and wound healing complications within the first 30 d after OLT[65]. Thus, sirolimus was not approved in liver transplantation, although recent studies using lower doses showed an improved safety profile[66,67]. Viceversa, everolimus provides a new therapeutic option for liver transplant recipients, when introduced early after liver transplantation[68] particularly with respect to posttransplant nephrotoxicity and other adverse events associated with long- term administration of CNIs.

Several studies[69-71] showed that hyperlipidemia was more frequent in the everolimus-treated patients

Table 2 Most used immunosuppressive drugs and main metabolic side effects

Factor	Metabolic consequences	Ref.
Steroid	Increased fat deposition with truncal fat distribution Decreased fat oxidation Increased gluconeogenesis Obesity Decreased glucose utilization Decreased b-cell insulin production Increased proteolysis, Reduced protein synthesis Insulin resistance Diabetes, NAFLD Mineralocorticoids effects Sodium retention Hypertension Hyperlipemia	[36,53-55]
Calcineurin inhibitor	Tacrolimus: b-cell toxicity Decreased insulin secretion Insulin resistance, Diabetes (more than cyclosporine) Cyclosporine: Decreased energy metabolism and muscle mass obesity Weight gain Decreased cholesterol transport into bile hyperlipidemia Occupy LDL receptor (more than tacrolimus) Renal vasoconstriction Hypertension (more than tacrolimus)	[58-65]
mTOR inhibitor	Increase insulin response Block b-cell proliferation Alter insulin signaling Decreased diabetes Increased diabetes Increased triglyceride production pathways And secretion Increased adipose tissue lipase activity Hyperlipidemia Decreased Lipoprotein lipase activity	[68,125-129]
Anti-metabolites	Mycophenolate mofetil: No nephrotoxity No effect on lipid profile, hypertention or diabetes mellitus Azatioprine: Vascular calcification Arteriosclerosis	[145-149]
Monoclonal antibodies	Basiliximab No nephrotoxity Rare effect on lipid profile, hypertension and diabetes mellitus	[150]

than in those treated with CNIs. The relationship between dyslipidemia during mTOR inhibitor administration and cardiovascular outcomes has not been systematically evaluated, and thus the clinical effect of these adverse events is not fully understood. However, the proportion of patients receiving lipid lowering treatment was similar when everolimus associated to a reduced doses of tacrolimus or the standard-of-care tacrolimus treatment were given[72]. Furthermore, the incidence of cardiovascular events after 24 mo did not differ between the two treatment groups[70]. The relationship between high rates of dyslipidemia and mTOR inhibitor use (sirolimus and everolimus), either in conjunction with or instead of CNIs, may be due to altered insulin signaling pathways that result in excess triglyceride production and secretion.

Thus, it is evident that the type of immunosuppressive therapy may strongly influence the occurrence of metabolic complications (Table 2).

NAFLD, A LEADING CAUSE OF OLT, ALSO COMPLICATES OLT DUE TO ITS FREQUENT RECURRENCE OR *DE NOVO* OCCURRENCE

In the last 10 years a marked increase of OLT for NASH cirrhosis was observed while that of HCV remained stable[36]. Thus NAFLD is expected to become the most

common indication for OLT over the next decade, given that HCV related morbidity will progressively decrease. Post-transplant NAFLD can be due to the recurrence of pretransplant MS and NAFLD, but often develops *de novo* because of modified metabolic conditions and use of the immunosuppressive drugs. NAFLD recurrence post-liver transplantation may progress to end-stage disease with liver failure and a need for retransplantation[73-76]. NAFLD incidence after liver transplantation ranges from 18 to 40% and that of NASH between 9%-13%. In addition, in patients transplanted for cryptogenic cirrhosis, the time-dependent risk of developing allograft steatosis is 100% over 5 years[40,75,77,78], indirectly confirming that cryptogenic cirrhosis is an evolution of NASH.

It is also worth noting that genetic background seems to play a role in allograft steatosis, since post-transplant NAFLD risk is linked to a polymorphism in adiponectrin (PNPLA3), which mediates triglyceride hydrolysis and has been reported to be the strongest genetic factor for liver steatosis, independently of insulin resistance. This polymorphism has been repeatedly reported to be associated with more severe fibrosis in patients with NASH and is also associated with pre-transplant obesity risk and presence of steatosis in the donor graft[79,80].

The natural history of post-transplant *de novo* NAFLD is poorly understood, but it may contribute to increased CVD mortality, since NAFLD is an independent risk factor for CVD even in non-cirrhotic patients[34,77,81].

It is very likely that the mediator of these processes is insulin resistance, which is linked to weight gain and high-dose steroid use post-transplantation, and is reflected by worsening of glucose tolerance, and underlies all manifestations of MS[76,81,82]. Overall, the main consequences of post-transplant MS appear to be NAFLD recurrence/development, higher incidence of adverse CVD events, and chronic transplant nephropathy[64].

Taken together, these evidence clearly demonstrate that a strict selection of patients with a complete cardiovascular assessment is necessary before listing patients for OLT in order to optimize resources and start early therapy to prevent complications.

CARDIOVASCULAR SCREENING PRE-OLT

In patients with cirrhosis, a clinical syndrome named cirrhotic cardiomyopathy has been noticed. This pathology is defined as a blunted contractile responsiveness to physiologic, pathologic, or pharmacologic stress and/or altered diastolic relaxation with electrophysiological abnormalities but with normal increased cardiac output and contractility at rest, in the absence of known cardiac disease and irrespective of the causes of cirrhosis[83]. Strict diagnostic criteria are lacking and this syndrome often goes unrecognized.

Van Wagner, as previously reported, showed that non coronary incidents represent the major adverse cardiovascular events after OLT, including atrial fibrillation, heart failure, thromboembolism and stroke[11].

This suggests that some OLT candidates may have subclinical CVD and may not be identified as patients at high risk when using standard risk algorithms. A study designed to evaluate the association between the presence of segmental myocardial perfusion defects pre-OLT by using myocardial perfusion scintigraphy and the occurrence of post-OLT complications and 1-year mortality after OLT, showed that even the presence of a single reversible perfusion defect was significantly related to an increased incidence of 1-year all-cause mortality. Due to these results the authors suggest the use of myocardial perfusion scintigraphy in the work up process[84]. Other studies point out the attention to pre-transplant pathology detected by echocardiography, including valve regurgitation, pulmonary artery pressure, right and left ventricular size, systolic function and left ventricular ejection fraction[85-87]. One study found a positive association between left ventricular hypertrophy and post-transplant death[85], whereas others yielded conflicting results regarding tricuspid regurgitation and post-transplant death[86,87].

Bushyhead *et al*[88] tried to determine if specific findings in pre-transplant echocardiography were associated with post-transplant survival and the development of cardiovascular and renal disease. The results of this study showed that increasing pulmonary artery systolic pressure was associated with significantly increased risk of hospitalization for myocardial infarction or heart failure, while increased left ventricular ejection fraction, a possible expression of cirrhotic cardiomyopathy, was associated with a non-significant increased risk of stage 4 or 5 chronic kidney disease.

Thus, because of the high risk of cardiovascular complications after OLT, careful preoperative evaluation of coronary risk is assessed in every transplant center. However, there is not yet a general agreement on a standard cardiovascular screening in OLT candidates. European guidelines suggest that electrocardiogram and echocardiography should be performed in all liver transplant candidates. If the patient has multiple cardiovascular risk factors, and is older than 50 years, a more extensive work up has to be assessed, including a cardiopulmonary exercise test to uncover asymptomatic ischaemic heart disease[89]. If the target heart rate is not achieved during a standard exercise test, a pharmacological stress test is the test of choice. If coronary disease is suspected, coronary angiography should be performed[90].

THERAPY AND FOLLOW UP AFTER LIVER TRANSPLANTATION

After OLT, to prevent cardiovascular events it is necessary to plan a follow up and a therapy focused on the control of metabolic syndrome manifestations, including control of blood pressure, blood glucose, lipid levels and weight, in addition to encouraging physical activity, and a correct diet. Individualized immunosuppressive therapy should also be designed. Furthermore, it is important to assess the presence of early vascular and cardiac damage, and to recognize their progression by carotid ultrasound and echocardiography, in order to be able to start specific therapy and prevent CV events in the future.

Given the high risk of developing NAFLD after OLT, therapy to prevent its occurrence and/or to treat it, if already developed, should be started. However, currently the only exploitable therapy for NAFLD is diet (Mediterranean diet is recommended[91]) and physical activity, although all available data suggest that improving insulin sensitivity could reduce the risk of post OLT NAFLD recurrence or *de novo* development. Drugs as thiazolidinediones (PPARγ agonist with insulin sensitizing effects), metformin, incretin-mimetics (liraglutide), antioxidants (vitamin E), angiotensin converting enzyme inhibitors have given promising results in patients with NAFLD. Several other pharmacological therapies for NAFLD are being studied, such as obeticolic (a syntetic farnesoid X receptor agonist), n-3 polynsaturated fatty acids (PUFA), and novel agents with anti-inflammatory, anti-fibrotic or insulin sensitizing properties [dual PPAR ά/δ agonists, dual chemokine receptor (CCR)2/CCR5 agonists and fatty acid/bile acid conjugates] and antifibrotic anti-lysil oxidase-like (anti-LOXL2) monoclonal antibodies[92]. While data on pentoxyphilline and orlistat have provided limited or inconclusive results, as well as those on lipid lowering drugs (ezetimibe and statins), no clinical trials have been conducted in the post-transplantation setting[34,76,82,93,94].

Thus, at present the only effective approaches for avoiding cardiovascular disease in the post-transplant setting are to prevent and manage MS and its manifestations. Table 3 presents the current available therapies to control metabolic syndrome manifestations.

Also renal dysfunction plays a relevant role in the occurrence of cardiovascular disease and death after transplantation. Therapeutic strategies should be focused to minimize renal injury, particularly in NAFLD patients, for example, by reducing exposure to CNIs[95]. This can be accomplished by reducing or withdrawing CNIs after the stable introduction of mycophenolate mofetil, introducing non-CNIs-based immunosuppressive protocols with mTOR inhibitors (sirolimus and everolimus) or reducing the CNIs dose in combination with mTOR inhibitors. The use of such protocols will require further prospective studies within the context of liver transplantation[95].

Problems that remain to be resolved

The onset of cardiovascular modifications after OLT remains poorly understood, the timing in which these modifications occur after OLT is still being debated. Only a few studies (based on paediatric population and prevalently on kidney transplant) and a meta-analysis[96-103] have shown that after solid organ transplantation there was a rapid increase of subclinical atherosclerosis evaluated by aortic stiffness and carotid intima-media thickness.

A recent study[104] demonstrated that at 1 year post-transplant, independently of the indication to OLT, LT recipients have similar pro-atherosclerotic profiles as patients with NASH, as measured by endothelial biomarkers and inflammatory cytokines, even when conventional cardiovascular risk factors, such as obesity or elevated Hs-CRP or/and high FRS, are not observed.

OUR PRELIMINARY RESULTS

We are conducting a prospective study aimed to understand the types of cardiovascular modifications and their time of development after OLT. Seventy-nine patients in a liver transplant list, were enrolled from 2014 and followed for 2 years after transplant. In these patients cardiovascular, biochemical and anthropometrical parameters were assessed at admission to the transplant waiting list, and at 6, 12, and 18 mo after transplant. The cardiovascular study included: evaluation of cIMT, presence of plaques by carotid ultrasound, diastolic function (E/A), interventricular septum, ventricular mass, and epicardial fat thickness evaluation by echocardiography. Preliminary data showed that cIMT progressively increased during follow up, starting as early as the 6th month, while prevalence of plaques was similar pre and post-transplant. A significant decrease of diastolic function (E/A) and an increase of inter-ventricular septum was observed from enrollment to 6 mo, which then remained stable over time. A progressive increase of epicardial fat was observed during follow up, while ejection function, and ventricular mass did not significantly differ. These preliminary results are shown in Figure 1.

It is yet to be determined if different immunosuppressive therapies influence these early changes and/or if other predisposing factors contribute to cardiac and vascular damage.

Future research directions that may maximize practical impact on the field

OLT candidates and recipients should be carefully evaluated and followed up not only for liver, but also for metabolic complications, optimizing the follow up

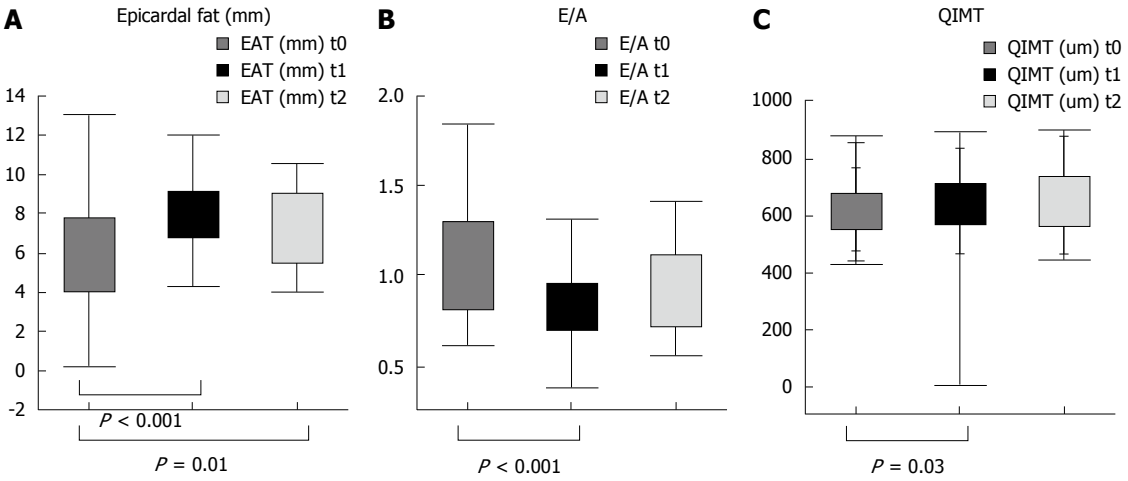

Figure 1 Modification of cardiovascular parameters during follow up: dark grey enrollment, black 6th mo, light grey 12th mo. A: Epicardial adipose thickness (EAT) significantly increased from baseline to 6th mo; B: Diastolic function (E/A) worsened significantly from baseline to 6th mo; C: Intima-media thickness (IMT) increased significantly from baseline to 6th mo.

Table 3 Post transplant metabolic syndrome manifestations and their possible therapy

Disease	Suggested therapy	Contraindicated therapy	Ref.
Diabetes mellitus	Insuline: in the early post-operative setting	Metformin: not usable with renal failure (lactic acidosis)	[130-133,151-157]
	Life-style modification (diet, physical activity)	Thiazolidinediones: may be associated to hepato and cardiotoxicity and are adipogenic	
	Oral hypoglicemic agent (after steroids tapering):	Second generation sulfonylureas: determine weight gain, hypoglycaemia, may increase CNI level	
	Metformin: less weight gain and hypoglicemia	Meglitinides: determine weight gain, hypoglycemia (only with renal insuff), CNI may increase repaglinide level, are expensive	
	Thiazolidinediones: well tolerated, may improve post-LT NAFLD	Alpha-glucosidase inhibitors: determine gastrointestinal side effects,are less effective, are expensive	
	Dypeptyl peptidase-4 (DPP4) inhibitors, well tolerate, no weight gain, no hypoglicemia, potential anti-inflammation, antihypertension, antiapoptosis effects and immunomodulation on the heart, vessels, and kidney, independent of their hypoglicemic effect	Selective renal sodium glucose co-transporter 2 (SGLT 2): dapagliflozin, canagliflozin, empagliflozin, well tolerated but reported hepato-toxicity, contraindicated in patients with renal impairment	
Hyperlipidemia	Hypercholesterolemia responds to:	Statins (except pravastatin and flestatin) are metabolized by cytochrome P-450 3A4, the same that metabolize CNIs and sirolimus so they must be used with caution because of myotoxicity	[134-138]
	HMGCoA inibitors (statins): pravastatine is the most studied and used but also atorvastatin, simvastatin, lovastatin, cerivastatin and fluvastatin are used	If used with statins fibrates may increase calcineurin inibitors levels	
	Diet rich in omega 3 fatty acids, fruits, vegetables and dietary fiber		
	Hypertrigliceridemia responds to:		
	Fish oil (omega 3)		
	Fibric acid derivates (gemfibrosil, clofibrate, fenofibrate)		
Arterial hypertension	First line agents: calcium channels blockers (amlodipine, isradipine, felodipine)	Nifedipine may increase CNI levels and may cause leg edema	[139-141]
	Second line agents: specific β-blockers, ACE inibitors, angiotensin receptors blockers and loop diuretics	ACE inibitors and angiotensin receptors blockers may exacerbate CNI-induced hyperkalemia, but may provide anti-fibrotic properties and possibly protect against calcineurin induced renal injury	
		Thiazides and other diuretics must be used with close follow-up because of potentiation of electrolyte abnormalities, hyperuricemia and renal dysfunction	
Obesity	Bariatric surgery: well tolerated and successful but require a complex reoperation	Orlistat (tetrahydrolipstatin), inhibitor of pancreatic lipase has limited efficacy and possibly interferes with immunosuppressive therapy	[141-144]
	Gastric banding at the time of liver transplant procedure seems successful and well tolerate	Gastric bypass surgery can affect intestinal drug absorption	

by introducing blood tests and imaging approaches that are able to show early metabolic, cardiovascular and atherosclerotic alterations.

New parameters that are able to identify subjects at higher metabolic/cardiovascular risk should be identified to plan personalized therapy, including nutritional rules and physical activity.

Prospective studies aimed to evaluate the development of early atherosclerotic damage are needed to understand the timing in which a specific therapy should be started.

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P- Reviewer: Keller F, Ramsay MA **S- Editor**: Qi Y **L- Editor**: A
E- Editor: Wang CH

World Journal of Gastroenterology

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Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.3748/wjg.v22.i40.8883

World J Gastroenterol 2016 October 28; 22(40): 8883-8891
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

REVIEW

Pathophysiological and clinical aspects of gastric hyperplastic polyps

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Author contributions: Markowski AR collected the information from published literature and wrote the paper; Markowska A collected the information from published literature and edited the manuscript; Guzinska-Ustymowicz K corrected the manuscript and designed the research.

Conflict-of-interest statement: There is no conflict of interest among the authors of this study.

Manuscript source: Unsolicited manuscript

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Received: May 28, 2016
Peer-review started: May 30, 2016
First decision: July 13, 2016
Revised: August 11, 2016
Accepted: August 23, 2016

Article in press: August 23, 2016
Published online: October 28, 2016

Abstract

Gastric polyps become a major clinical problem because of high prevalence and tendency to malignant transformation of some of them. The development of gastric hyperplastic polyps results from excessive proliferation of foveolar cells accompanied by their increased exfoliation, and they are macroscopically indistinguishable from other polyps with lower or higher malignant potential. Panendoscopy allows detection and differentiation of gastric polyps, usually after obtaining histopathological biopsy specimens. Unremoved gastric hyperplastic polyps may enlarge and sometimes spontaneously undergo a sequential progression to cancer. For this reason, gastric hyperplastic polyps larger than 5 mm in size should be removed in one piece. After excision of polyps with atypical focal lesion, endoscopic surveillance is suggested depending on histopathological diagnosis and possibility of confirming the completeness of endoscopic resection. Because of the risk of cancer development also in gastric mucosa outside the polyp, neighboring fragments of gastric mucosa should undergo microscopic investigations. This procedure allows for identification of patients who can benefit most from oncological endoscopic surveillance. If *Helicobacter pylori* (*H. pylori*) infection of the gastric mucosa is confirmed, treatment strategies should include eradication of bacteria, which may prevent progression of intestinal metaplasia. The efficacy of *H. pylori* eradication should be checked 3-6 mo later.

Key words: Gastric hyperplastic polyp; Pathophysiology; Gastric cancer; Surveillance

Core tip: The present review is one of only a few papers describing the clinical problem of gastric hyperplastic polyps and their tendency to malignant transformation. For this reason, gastric hyperplastic polyps larger than 5 mm in size should be removed, preferably in one piece. After excision of polyps with dysplasia, careful endoscopic surveillance is needed, both places after polypectomy and surrounding mucosa.

Markowski AR, Markowska A, Guzinska-Ustymowicz K. Pathophysiological and clinical aspects of gastric hyperplastic polyps. *World J Gastroenterol* 2016; 22(40): 8883-8891 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i40/8883.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i40.8883

INTRODUCTION

A polyp is a proliferative or neoplastic lesion of the mucous membrane, directed toward the gastrointestinal lumen, projecting from the surrounding mucosa, and having the head and (sometimes) the stalk[1]. Some gastric polyps tend to have malignant transformation to cancer and gastric cancer is the third most common cause of cancer-related death in the world and being still difficult to cure because of advanced disease at the moment of diagnosis.

Gastric polyps are detected during 1%-6% of upper gastrointestinal endoscopies and in 0.1%-0.8% of autopsies[2-4]. Still several years ago, gastric polyps were twice more frequent in the antrum than in the body of the stomach. It seems, however, that their location has changed in the past 10 years; the incidence of polyps has increased in the stomach body (19% *vs* 32%) and decreased in the antrum (46% *vs* 24%)[3]. Also, altered age distribution of gastric polyps was observed in the last decade; patients aged 45-59 have currently twice more gastric polyps than 10 years ago, but the inverse relationship is observed for patients aged 60 years and over[5].

According to the macroscopic classification of Yamada and Ichikawa, polyps can be divided into: 1/flat polyps, *i.e.,* slightly elevated and with indistinct margins, height < 2.5 mm (width of closed biopsy forceps), 2/sessile polyps, *i.e.,* elevated with a distinct border at the base, yet without a notch, height exceeds 2.5 mm, 3/semi-pedunculated polyps, *i.e.,* elevated with distinct margins and clear notch at the base, but without peduncle and 4/pedunculated polyps[6]. Gastric epithelial polyps include fundic gland polyps, hyperplastic polyps and adenomatous polyps[7,8].

Gastric hyperplastic polyps (GHPs) can be single (68%-75%) or multiple, they occur sporadically (isolated polyps) or as a component of a rare hyperplastic polyposis syndrome (the presence of 50 or more polyps). Sporadic GHPs are macroscopically and histologically indistinguishable from the syndromic GHPs and the latter are associated with a higher risk of malignant transformation and higher 5-year mortality rate. Solitary GHPs with distinct margin, red color and protuberant shape can be difficult to distinguish from well-differentiated adenocarcinoma. Sometimes GHPs are found to coexist with other types of polyps or tumors (synchronous polyps)[2,9,10]. Geographical differences exist in the prevalence of gastric polyps. In Western countries, the incidence of GHPs decreased[2], while in areas where the prevalence of *Helicobacter pylori* (*H. pylori*) is high, GHPs have been reported more frequently[11].

PATHOPHYSIOLOGY

It is believed that GHP development results from excessive proliferation of foveolar cells (mucin-producing epithelial cells lining the gastric surface and the gastric pits) accompanied by their increased exfoliation; glands are usually not involved in the formation of polyps. Stimuli directly responsible for the appearance of GHPs have not been known yet, although it seems that abnormal hyper-regenerative processes triggered in response to nonspecific gastric mucosal injury can be suspected. Two main pathologies lie in the background of GHPs: longstanding *H. pylori*-associated gastritis and autoimmune metaplastic atrophic gastritis in Addison-Biermer disease; GHPs are less common in other inflammatory conditions, in the vicinity of ulcers, erosions and surgical gastroenterostomy, secondary to prior endoscopic coagulation therapy, in gastric mucosa with slight atrophy or metaplasia, and in cardia in patients with gastrointestinal reflux. GHPs almost never occur in normal gastric mucosa.

The risk of developing GHPs increases with the degree of mucosal atrophy, especially when the stomach body is affected. Over time, GHPs may remain stable, increase in size, or even regress. It was not long ago that GHPs were believed to be benign lesions not associated with the risk of malignant transformation. Today, however, unremoved GHPs are known for their ability to enlarge and sometimes spontaneously undergo a sequential progression and a few-phase neoplastic transformation[12]. This process has been confirmed and well documented in GHPs, although it is much less common than in adenomatous polyps of the stomach.

Although Helicobacter pylori is sometimes present within GHPs[13], the bacterium not induces specifically their growth or malignant transformation[14]. It is estimated that most of GHPs remain stable in time, but 27% may enlarge[15]. It appears that, in addition to age, there are known some clinical factors predicting for the possibility of neoplastic transformation of GHPs, such as polyp size (greater than 1 cm), pedunculated morphology, postgastectomy state, and synchronous neoplastic lesion[16,17].

Early gastric cancer in gastric hyperplastic polyps, by

definition, does not infiltrate deeper than the submucosa, irrespective of local lymph node involvement. In the pedunculated polyps, the submucosal layer ascends through the stalk to the head, while in sessile ones, the submucosal layer forms convexity toward the tumor. The depth of penetration can be assess only when the cross-sectional image perpendicular to the lesion and contiguous normal wall are obtained. The prevalence of gastric cancer in GHPs shows a positive correlation with the polyp size, whereas mortality due to GHPs depends on the presence and severity of neoplastic transformation in the polyp[18]. Gastric metastases are relatively rare, but even a case of metastasis to GHP has been recently described to[19].

EPIDEMIOLOGY AND SYMPTOMATOLOGY

Although the existing epidemiological data are not explicit, the multi-center research trial conducted in the United States to assess so far the largest number of panendoscopies (120000) and gastric polyps (5877) suggests that the most common types of gastric polyps are gastric fundic gland polyps (77%) and GHPs (14%); much less common are: polypoid foveolar hyperplasia (2.7%), adenocarcinomas (1.3%), lymphomas (0.9%) and adenomas (0.7%)[2]. A recently published retrospective study which assessed the United States national database of histopathological reports involving approximately 741000 patients, confirmed the highest prevalence of gastric fundic gland polyps and hyperplastic polyps, 7.72% and 1.79% of patients undergoing gastroscopy, respectively[20]; adenomatous polyps of the stomach were much less common. In other clinical studies, depending on the definition and histopathological criteria, the study period and population, GHPs accounted for 25%-28% to 71%-76% of all gastric polyps[4,21-28].

The incidence of GHPs increases with age and although they can also be found in children, GHPs usually affects the 65-75 year-old population[1]. Most studies proved higher incidence of all types of gastric polyps in women than in men[1,3,22]. In a study performed by Cao *et al*[3] gastric polyps were found in 34% of men and 66% of women (24121 patients). However, it was the result of higher prevalence of gastric fundic gland polyps in women (43% *vs* 55%), since the prevalence of gastric hyperplastic polyps was similar in both genders (27% *vs* 29%). Besides, adenomatous polyps, which were much less common, were more often observed in men (15% *vs* 4%). Although the percentage of all gastric polyps found during panendoscopies has not changed in the last decade, it seems that the relative incidence of GHPs showed a twofold decrease, which was accompanied by a substantial increase in the relative incidence of gastric fundic gland polyps. It is speculated that this phenomenon can be the effect of a common use of proton pump inhibitors[3].

Gastric traditional serrated adenomas (TSA) were described for the first time in 2001. A novel histologic phenotype of gastric adenoma are characterized by protruding glands with lateral saw tooth-like notches due to scalloped epithelial indentations; gastric TSA have emerged as very aggressive, because nearly 75% of them exhibited invasive carcinoma[29].

GHPs are usually asymptomatic and therefore incidentally found during panendoscopies performed for various reasons[2]. Symptoms due to GHPs are nonspecific: dyspepsia, heartburn, bleeding from the upper GI tract (usually latent), and sometimes gastric outlet obstruction. Only sideropenic anemia can be an indirect nonspecific presentation of a large and fragile GHPs. Imaging diagnostic examinations (X-ray with contrast agent, computed tomography) have little significance due to high false-negative rates; they can sometimes reveal only large GHPs. Panendoscopy is the investigation of choice allowing detection and differential diagnosis of gastric polyps, usually after obtaining histopathological biopsy specimens.

MACROSCOPIC AND HISTOPATHOLOGICAL PICTURE

GHPs are usually small, flat or sessile dome-shaped lesions with smooth surface and lobular structure (Figure 1). The proportional prevalence of GHPs according to size is estimated at: 47% (< 0.5 cm), 25% (0.6-0.9 cm), 18% (1-2 cm), 6% (2-3 cm) and 4% (> 3 cm)[30]. Sometimes GHPs may have erosions on their surface and they are often difficult to distinguish from polypoid foveolar hyperplasia or gastric adenomatous polyps[1]. Sometimes GHPs are very big and have aciniform structure. They may reach even 13 cm in size and then they resemble a neoplastic tumor. A large size of gastric hyperplastic polyps and granular structure with visible depression and mucus threads on the surface may suggest their malignant transformation.

Endoscopy with optic image magnification and NBI allows the assessment of the network of fine blood vessels, which correlates well with histopathological findings and increases the possibility of early differentiation of gastric polyps already during endoscopy; dense distribution of irregular capillaries on the polyp surface is characteristic of GHPs[31].

Contrary to hyperplastic polyps of the colon, GHPs show swelling of the submucosal membrane with pronounced foveolar hyperplasia and infiltration of the lamina propria by inflammatory cells, among which smooth muscle cells derived from thickened and cracked muscle membrane can be seen. Mucin-secreting cells from the foveolar layer of GHPs are enlarged and elongated; they form canals that extend to the stroma, which can enlarge and form marked irregular cysts varying in shape and size. PAS/Alcian

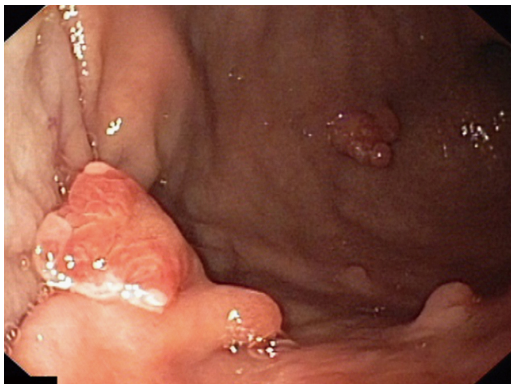

Figure 1 Endoscopic view. Large gastric hyperplastic polyp.

blue or mucicarmine stains highlight acidic mucin in goblet cells and can demonstrate the neutral mucin in foveolar epithelium[10].

GHPs have two major and typical microscopic features[10]. The first and salient includes distinctly elongated, dilated, distorted and branched pits of the mucosa, with a folded epithelial lining (differing in height) that does not exfoliate in proper time (Figure 2A-C). This leads to increased mucus secretion and a spiral appearance of the mucosal pits on the horizontal section or serrated and star-like appearance on the cross-section[18]. The foveolar cells mature excessively, contain large amounts of cytoplasm and small nuclei, and exhibit low mitotic activity[21]. The swollen stroma shows a network of randomly arranged, diffused fine bundles of smooth muscles, located in the lamina propria. The glandular epithelium can sometimes occur only in deeper layers of the polyps.

The second typical microscopic feature is swelling and inflammatory reaction of the stroma of varied intensity, either acute or chronic, *i.e.,* visible infiltration of the lamina propria by numerous neutrophils, plasmatic cells, lymphocytes, eosinophils, mastocytes and macrophages. These regions are strongly vascularized and vascular proliferation resembles granulation. Because of local trauma, the surface of GHPs can be ulcerated and inflamed, with regenerative atypia of epithelial and interstitial cells (Figure 2D). Sometimes the surface of the mucous membrane can also exhibit budding pits having features of pseudo-invasion. For a histopathologist, such lesions are a major diagnostic issue, since hyperplastic polyps may sometimes contain foci of dysplasia and cancer. Abnormal regenerative changes may be difficult to differentiate accurately from dysplastic atypia[32,33].

If dysplasia within GHPs is confirmed by biopsy, it is crucial to determine its grade and boundaries, and assess whether it is limited only to the polyp or is just a fragment of the extensive neoplastic process. If dysplasia develops only in the polyp and its focus is removed radically during polypectomy, both macroscopically and microscopically, the lesion is considered to be cured.

DIFFERENTIAL DIAGNOSTICS

GHPs should be differentiated from other sporadic polyps (fundic gland polyps, adenomatous polyps) and lesions of the mucosa present in familial polyposis syndrome (Ménétrier disease, juvenile polyposis and Cronkhite-Canady syndrome). Earlier clinical studies suggesting high incidence of GHPs launched a debate on diagnostic criteria and the factual incidence rate. It seems that most of the previously described tiny hyperplastic polyps were in fact only the hyperplasia of the foveolar layer of the gastric mucosa. Polypoid foveolar hyperplasia (PFH) is regarded as a precursor of gastric hyperplastic polyps and differs slightly from them in the microscopic structure. Elongated pits of the mucosa but without features of dilatation can be also seen in PFH, and the lamina propria is either normal or only slightly swollen[2]. Differentiation between these two lesions is of crucial clinical significance since malignant transformation affects gastric hyperplastic polyps but not foveolar polypoid hyperplasia[24]. Precise categorization of gastric polyps is being conducted. Multicenter clinical study results published in 2011, revised the previous histopathological assessment of gastric hyperplastic polyps using precise diagnostic criteria; only in 20% of cases, previous diagnosis of GHP was confirmed[24].

CLINICAL SIGNIFICANCE OF INTRAEPITHELIAL NEOPLASIA IN THE MUCOSA SURROUNDING A POLYP

Oncological risk associated with GHPs depends on the risk of cancer development not only in the polyp, but also in gastric mucosa outside the polyp. Thus, also the neighboring fragments of gastric mucosa should undergo endoscopic and microscopic investigations.

The risk of focal gastric cancer is five-fold higher in gastric adenomatous polyps than in the hyperplastic ones (10% *vs* 2.1%), and 2-fold higher in gastric mucosa surrounding the adenomatous than hyperplastic polyps (13.3% *vs* 7.1%). Thus the risk of cancer growth in gastric mucosa outside the polyp is probably slightly higher than in the polyp itself[21].

Gastric hyperplastic polyps are frequently associated with inflammatory lesions in the local gastric mucosa. Chronic inflammation of gastric mucosa can be observed in associations with *H. pylori* infection (25%), autoimmune inflammation (12%), atrophic gastritis, lymphocytic inflammation or CMV infection[30]. In a patient with GHPs the Sydney biopsy protocol recommends collection of five separate specimens: two from the stomach body (greater and lesser curvature), two from the antrum (greater and lesser curvature) and one from the gastric angle. When *H. pylori* infection of the gastric mucosa is confirmed, treatment of small GHPs should begin from eradication therapy, which in many cases reduces or eliminates

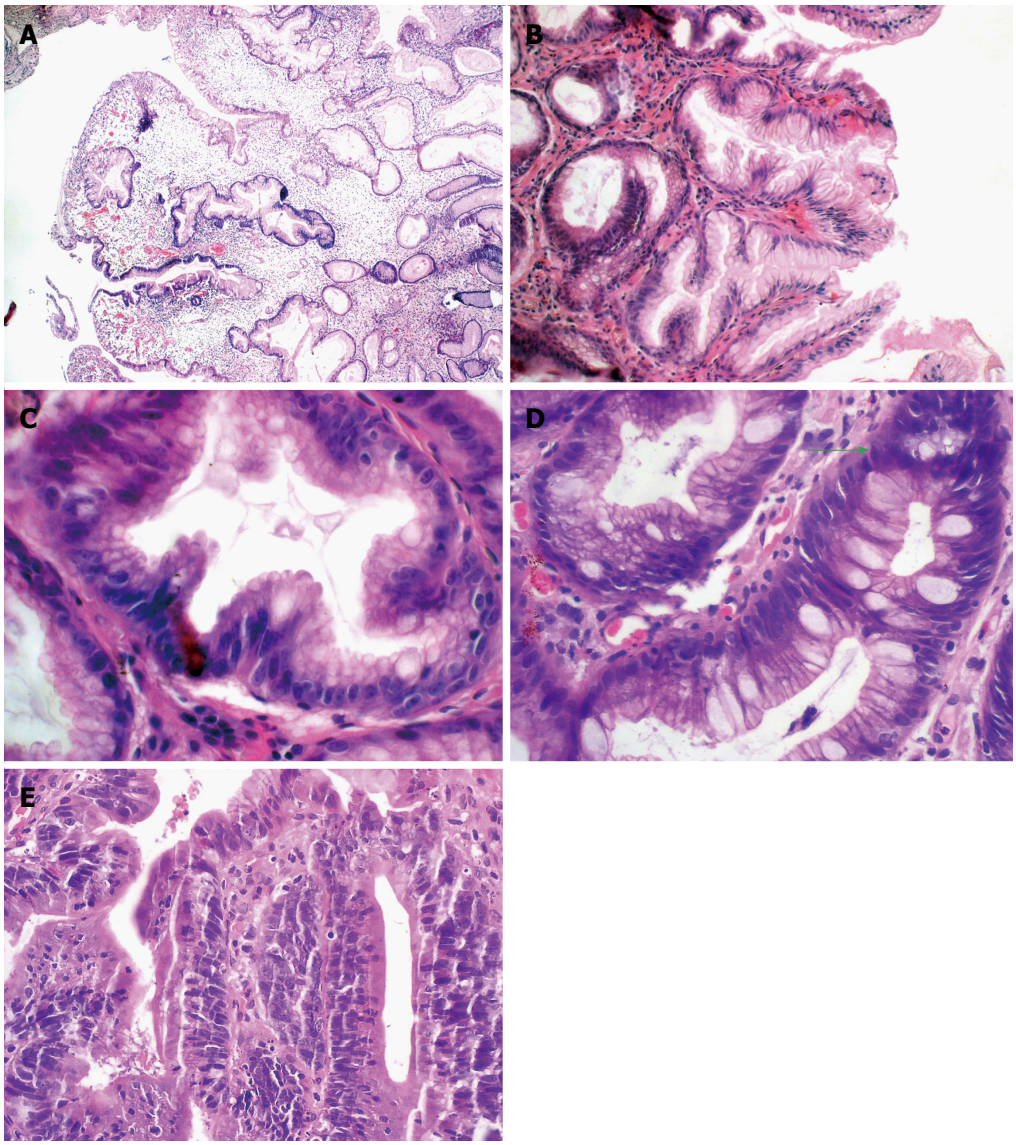

Figure 2 Histopathological findings. A: Gastric hyperplastic polyp with dilated, elongated, branched and foveolar epithelium and edematous end inflamed stroma (original magnification × 10); B: Gastric hyperplastic polyp with well visible elongated foveolar epthelium (original magnification × 20); C: A cross-section of mucosal crypt shows a serrated ligh of the gland and the goblet cells (original magnification × 40); D: The green arrow indicates a regeneration zone in the foveolar epthelium with hyperchromatic nuclei (original magnification × 40); E: Focus of adenocarcinoma in the gastric hyperplastic polyp (original magnification × 40). Hematoxylin-eosin staining.

the polyps[34]. The efficacy of such treatment should be checked 3-6 mo later.

If the gastric mucosa surrounding a hyperplastic polyp exhibits features of chronic atrophic gastritis, its stage should be examined using the OLGA system (Operative Link on Gastritis Assessments), allowing better stratification of the risk and identification of patients who can benefit most from oncological endoscopic surveillance. It seems that patients with diffused atrophy should be included in such a program and have panendoscopy performed at first each year (OLGA IV), and then every 2 years (OLGA III) or every 5 years (OLGA II)[35].

The mucosa surrounding the hyperplastic polyps frequently shows chronic inflammation and sometimes oncologically hazardous focal intestinal metaplasia (37%) and dysplasia (2%) or even adenocarcinoma (6%)[30].

CLINICAL SIGNIFICANCE OF INTRAEPITHELIAL NEOPLASIA IN THE POLYP

Only a small percentage (< 2%-3%) of GHPs, usually the larger ones (> 1-2 cm), show features of focal intraepithelial neoplasia (IEN) or cancer (Figure 2E). Therefore, large polyps should be removed and as a whole subjected to histopathological analysis. Studies assessing the presence of intraepithelial neoplasia and adenocarcinoma in GHPs are rare[2,21,30,36-38].

The p53 protein, a *p53* suppressor gene product,

inhibits neoplastic transformation (by prolonging G1 phase of the cellular cycle), which gives the cells enough time to repair damaged DNA threads. If the damage is severe and cannot be quickly repaired, p53 initiates the process of programmed cell death. Mutations of *p53* gene result in the synthesis of mutated, *i.e.,* functionally abnormal p53, deprived of the inhibitory function, which promotes transfer of genetic disorders to daughter cells and facilitates neoplastic transformation. The half-life period of normal p53 is approximately 20 min only, whereas pathological p53, being the product of the mutated *p53* gene, shows a prolonged half-life, is accumulated in the cell and can be then easily detected.

The assessment of cell proliferation is a useful marker in the diagnosis of neoplastic transformation. Ki-67 antigen observed during all active phases of the cell cycle but absent from the G0 phase is presented as the percent of marked cell nuclei. It is a widely accepted marker of proliferation; the higher the expression of Ki-67, the higher malignancy grade.

In the histopathological material including 497 GHPs collected from 412 patients during an 11-year-period, the prevalence of intestinal metaplasia, dysplasia (intraepithelial neoplasia) and cancer within GHPs was estimated at 5%, 10% and 2.2%, respectively[38]. Positive expression of p53 and high proliferation index (mitotic index) of Ki-67 were observed in cases of focal intraepithelial neoplasia (41%) and cancer (50%) within GHPs, as compared to the hyperplastic regions and metaplastic foci[33,38]. The foci of intraepithelial neoplasia were always found close to the foci of adenocarcinoma. The research seems to confirm the theory of neoplastic transformation in hyperplastic polyps of the hyperplasia-dysplasia-adenocarcinoma type. The expression of certain membrane proteins called claudins (Cld) that are found in tight intracellular junctions and are responsible for cell membrane integrity is one of the markers of malignant transformation within hyperplastic polyps of the stomach, since the expression of Cld-3 has been demonstrated only within the foci of intraepithelial neoplasia and cancer[16].

Orlowska *et al*[21] estimated the prevalence of metaplasia, intraepithelial neoplasia (dysplasia) and cancer in GHPs to be 5.6%, 3.3% and 2.1%. In their research[21] and in a study conducted by Abraham *et al*[30], the percentage of intraepithelial neoplasia in hyperplastic polyps of the stomach is 10 times higher (3.3%-4% *vs* 0.4%) than in the study by Carmack *et al*[2], and in the study by Terada[38] even 25 times higher (10% *vs* 0.4%); perhaps, these authors were dealing with specially selected patients.

The risk of developing cancer in GHPs increases with the polyp size and is believed to be higher for GHPs > 2 cm, although cases of cancer in 5-10 mm polyps have also been described. Neoplastic transformation usually starts from a small focus of intraepithelial neoplasia, which grows and acquires features of invasiveness. Intraepithelial neoplasia involves cytological and architectonic disorders within the cell: changes in the nucleus-cytoplasm ratio in favor of the nuclei, increased number of mitoses in enlarged nuclei, excessive number of epithelial cells with their build-up and loss of nuclear polarization (hyperchromatic cell nuclei lose their parallel arrangement). Mild and severe intraepithelial neoplasia can be distinguished, depending on impairment severity.

TREATMENT

The management of gastric polyps depends on the clinical condition of the patient, malignant potential of detected polyps and at which stage of malignant transformation polyps have been found. Endoscopic removal of adenomatous or hyperplastic polyps, symptomatic or with dysplastic foci, is recommended if it is possible and safe. Studies comparing biopsy findings with histopathological assessment of radically removed polyps have shown approximately 90% compatibility. When the polyp is removed in one piece with a diathermic loop it is less probable that some advanced dysplastic and neoplastic lesions can be missed and more likely that total removal is accomplished; piecemeal polypectomy technique does not ensure radical removal. Biopsy of gastric mucosa outside the polyp and examination for *H. pylori* infection and its eradication are additionally recommended, with a single endoscopic check-up one year later[7]. At present, repeat endoscopic examinations of GHPs negative for intraepithelial neoplasia are not recommended (Table 1).

Polypectomy is indicated for all gastric polyps > 10 mm, to eliminate sampling error by missing any neoplastic foci and prevent neoplastic transformation. Periodic biopsies of polyps that are not classified for removal due to their size, number and the risk of postsurgical complications should be performed[25]. When multiple polyps occur, it is recommended to obtain bioptates or remove the largest polyp as well as obtain biopsy specimens from the remaining polyps. And then, decision for polypectomy should be made based on histopathology findings. Most of GHPs can be detected and treated using endoscopy alone. According to current recommendations, GHPs > 5 mm should be removed whole[8,17,18,39], especially the pedunculated ones[17].

Some researchers, however, considering the risk of the procedure (bleeding, perforation), suggest the removal of only large GHPs, in which the probability of intraepithelial neoplasia and cancer is the highest. In the case of multiple hyperplastic polyps without foci of intraepithelial neoplasia, conservative management and follow up endoscopy seems to be safer strategy than numerous polypectomies, although there are no reliable studies to support this suggestion.

Oncological surveillance of patients with hyperplastic

Table 1 The proposed management decisions and oncologic surveillance program regarding gastric hyperplastic polyps

Before endoscopic resection of GHPs
GHP without dysplasia or cancer, asymptomatic and small (< 5 mm) - surveillance not recommended
GHP symptomatic or larger than 5 mm - endoscopic resection recommended
GHP with dysplasia or cancer - endoscopic or surgical resection recommended
GHP not classified for removal due to the risk of postsurgical complications - periodic gastroscopies with representative biopsies every 1-2 yr
GHP in patients with high risk of gastric cancer[1] - gastroscopies every 1-2 yr
GHP with dysplasia outside the polyp - consider subtotal gastrectomy and gastroscopies every 1-3 yr
After endoscopic resection of GHPs
After complete resection of GHP with dysplasia - gastroscopy 1 yr later, and then depending on the clinical situation
After complete resection of GHP with early gastric cancer - gastroscopy 1 yr after and then 3 yr after
After incomplete resection of GHP with gastric cancer - consider gastrectomy with lymphadenectomy

[1]Family history of gastric cancer or OLGA 3-4 on histopathological examination. GHP: Gastric hyperplastic polyp.

polyps containing foci of dysplasia and cancer should be patient-tailored, since there are no generally accepted guidelines. It seems that when cancer was detected early in an endoscopically radically resected polyp, the oncological surveillance should involve repeated endoscopy, at the same frequency than for adenomatous gastric polyps, *i.e.,* first one year after and then 3 years after the procedure[17,39].

Endoscopic treatment of the polyp containing cancer is considered sufficient if it has been completely resected according to the endoscopist (macroscopic radicality) and histopathologist (microscopic radicality). If the cancer does not exceed the gastric mucosa, the excision margin free of cancer cells is greater than 2 mm in the microscopic investigation, differentiation degree of the cancer is high or moderate and no angioinvasion is observed, the resection is approved oncologically radical. The percentage of cancer relapse after radical resection of gastric hyperplastic polyps containing focal cancer is unknown, although it is certainly lower than that after the endoscopic resection of nonpolypoid early gastric cancer (1.2%)[40].

If incomplete resection of hyperplastic polyp containing early gastric cancer is evidently confirmed or when effective endoscopic treatment is impossible, we have to consider gastrectomy with local lymphadenectomy[41]. In surgical treatment of early gastric cancer, conventional open laparotomy is increasingly more often replaced by low-invasive surgical techniques.

The surveillance of malignant GHPs following endoscopic removal is difficult because of the possibility of residual neoplastic cells within the stomach wall. It is commonly believed that the patient with diagnosed cancer or high grade dysplasia in the polyp should be treated by a multi-specialist team dealing with the diagnosis of the upper GI tract. The diagnosis should be established by two pathologists, with at least one specializing in gastrointestinal diseases. The strategy of management and therapy should be discussed with the patient and experienced endoscopists should perform surveillance panendoscopy.

When the resected GHPs are free of dysplasia and cancer, the management of patients should depend on the risk of developing cancer assessed on the basis of the presence of chronic atrophic gastritis and/or other risk factors. Last data (405211 patients) predict that about 1 in 256 people with normal gastric mucosa, 1 in 85 with gastritis, 1 in 50 with atrophic gastritis, 1 in 39 with intestinal metaplasia, and 1 in 19 with dysplasia will develop gastric cancer within 20 years after gastroscopy[42].

In patients with high risk of gastric cancer (OLGA 3-4), with moderate or high grade diffuse atrophy of the mucosa, usually with enhanced metaplasia, in patients with a family history of gastric cancer, gastroscopies are recommended regularly, every year or every two years. Patients with low risk of gastric cancer (OLGA 1-2) should have at least one gastroscopy within 3-6 mo after the procedure, to confirm eradication of *H. pylori* and exclude the presence of new or residual polyps that would have to be removed.

When dysplasia is present in the mucous membrane of the stomach outside the polyp, especially in diffuse lesions, subtotal gastrectomy should be considered with postsurgical endoscopic surveillance, during which numerous bioptates are collected from the mucosa of the stomach stump at 1-3 year-intervals to exclude multifocal lesions[17,40].

CONCLUSION

Recent studies have confirmed that cancer may arise within GHP, and a malignant lesion is likely to take a hyperplasia-dysplasia-adenocarcinoma course. Polypectomy of GHPs > 5 mm is recommended, with histopathological diagnosis, and periodic biopsies of the polyps which are not qualified for removal should be obtained. Additionally, biopsy of gastric mucosa outside the polyp is indicated as well as *H. pylori* eradication in the case of confirmed infection. Endoscopic check-up is suggested a year after removal of dysplasia-free GHP. The surveillance of patients after polypectomy of GHP containing foci of dysplasia and cancer should be more intensive and individual; precise guidelines do not exist.

Extensive knowledge concerning the mechanisms of origin, malignancy potential, diagnostic possibilities

and GHP management may increase the efficacy of treatment of gastric polyps in everyday clinical practice.

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P- Reviewer: Chung JW, Dhalla SS, Garcia-Olmo D, Porumb V
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World Journal of Gastroenterology

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Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.3748/wjg.v22.i40.8892

World J Gastroenterol 2016 October 28; 22(40): 8892-8904
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

REVIEW

Indications and surgical options for small bowel, large bowel and perianal Crohn's disease

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Author contributions: The manuscript was prepared by Toh JWT and was reviewed and revised by Stewart P, Rickard MJFX, Leong R, Wang N and Young CJ.

Conflict-of-interest statement: The authors have no conflicts of interest to declare (refer to signed statement by the corresponding author).

Manuscript source: Invited manuscript

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Received: July 14, 2016
Peer-review started: July 16, 2016
First decision: August 19, 2016
Revised: August 26, 2016
Accepted: September 28, 2016
Article in press: September 28, 2016
Published online: October 28, 2016

Abstract

Despite advancements in medical therapy of Crohn's disease (CD), majority of patients with CD will eventually require surgical intervention, with at least a third of patients requiring multiple surgeries. It is important to understand the role and timing of surgery, with the goals of therapy to reduce the need for surgery without increasing the odds of emergency surgery and its associated morbidity, as well as to limit surgical recurrence and avoid intestinal failure. The profile of CD patients requiring surgical intervention has changed over the decades with improvements in medical therapy with immunomodulators and biological agents. The most common indication for surgery is obstruction from stricturing disease, followed by abscesses and fistulae. The risk of gastrointestinal bleeding in CD is high but the likelihood of needing surgery for bleeding is low. Most major gastrointestinal bleeding episodes resolve spontaneously, albeit the risk of re-bleeding is high. The risk of colorectal cancer associated with CD is low. While current surgical guidelines recommend a total proctocolectomy for colorectal cancer associated with CD, subtotal colectomy or segmental colectomy with endoscopic surveillance may be a reasonable option. Approximately 20%-40% of CD patients will

need perianal surgery during their lifetime. This review assesses the practice parameters and guidelines in the surgical management of CD, with a focus on the indications for surgery in CD (and when not to operate), and a critical evaluation of the timing and surgical options available to improve outcomes and reduce recurrence rates.

Key words: Surgery; Crohn's disease; Major abdominal surgery; Perianal; Inflammatory bowel disease; Colon cancer

Core tip: Despite significant advances in the medical management of Crohn's disease (CD), most patients will still need surgery during their lifetime, with a third requiring multiple surgeries. It is important to optimise the surgical management of CD in order to reduce rates of emergency surgery, surgical recurrence and intestinal failure. Surgical options depend on the phenotype of CD. The most common indications for surgery include stricturing disease, fistulae and abscesses whereas surgery for bleeding and cancer associated with CD is less common. It is vital to understand the role and timing of surgery, and the best surgical options in the management of CD.

Toh JWT, Stewart P, Rickard MJFX, Leong R, Wang N, Young CJ. Indications and surgical options for small bowel, large bowel and perianal Crohn's disease. *World J Gastroenterol* 2016; 22(40): 8892-8904 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i40/8892.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i40.8892

INTRODUCTION

Seventy to ninety percent of patients with Crohn's disease (CD) will eventually need surgery[1]. The likelihood of surgery in CD after initial diagnosis has been reported in a recent systematic review to be 16.3% at one year, 33.3% at three years and 46.6% at 5 years[2]. The surgical mortality rate is 0%-8.4%[3-5], with most deaths resulting from intra-abdominal sepsis. Absolute indications for surgery in CD include cancer, perforation, toxic megacolon and major life-threatening gastrointestinal tract (GIT) bleeding. Relative indications include strictures, phlegmon, fistulae, intra-abdominal abscesses, GIT bleeding, dysplasia-associated lesion or mass (DALM), high grade dysplasia detected on surveillance, growth retardation in children and failure of medical therapy. The decision of when to operate is difficult in CD. Delaying surgery for prolonged medical management may increase complication rates as well as increasing the technical difficulties encountered during surgery and the rates of emergency surgery, which is associated with increased stoma rates and at least a three-fold increase in mortality compared to elective surgery.

CD is associated with high surgical recurrence, such that most patients with CD will have multiple operations during their lifetime and 5%-18% of patients eventually require parenteral nutrition for intestinal failure[6,7]. The risk of intestinal failure rises significantly with multiple surgeries, particularly when intestinal length is shorter than 150 cm[7] and is imminent when intestinal length is less than 100 cm[8]. This review assesses the available evidence in the current literature on the surgical management of CD, focusing on the indications for surgery (Figure 1) and the surgical options for small bowel, large bowel and perianal CD.

LITERATURE SEARCH

The Preferred Reporting Item for Systematic Reviews and Meta-analyses (PRISMA) guidelines was used in this review. Two databases (MEDLINE and Embase from 2010-2016). Search terms included "Crohn's disease", "perianal", "large bowel" or "large intestines" or "colon", "small bowel" or "small intestines" and "surgery" or "surgical indications". Five hundred and ten studies were identified through MEDLINE and Embase, 189 additional studies were found from hand-searching references.

Abstracts were reviewed by two investigators independently, and only studies excluded by both investigators were excluded. When only one investigator excluded the study, these studies were included for full text review. Studies excluded based on abstract included non-human studies, non-English language, studies on immunomodulators and biological agents only, no reference to surgery for CD, reference to ulcerative colitis only, upper gastrointestinal CD only or inflammatory bowel disease in general but not specifically CD. Studies reporting mainly on immunomodulators but with references to surgery and studies on inflammatory bowel disease with reference to CD were included for full text review. Only studies which reported on indications and surgical options for small bowel, large bowel or perianal CD were eligible for qualitative synthesis. One hundred and twenty-eight full text studies were reviewed after duplicates were removed. One hundred and fifteen studies were included in this review.

INDICATIONS FOR MAJOR GASTROINTESTINAL SURGERY IN CD

Stricture and obstruction

Bowel obstruction from stricture (Figure 2) is the most common reason for surgery in CD[9]. Stricturing phenotype of CD is most common in ileal disease and in patients diagnosed with CD at a younger age. Patients may also develop anastomotic fibrotic

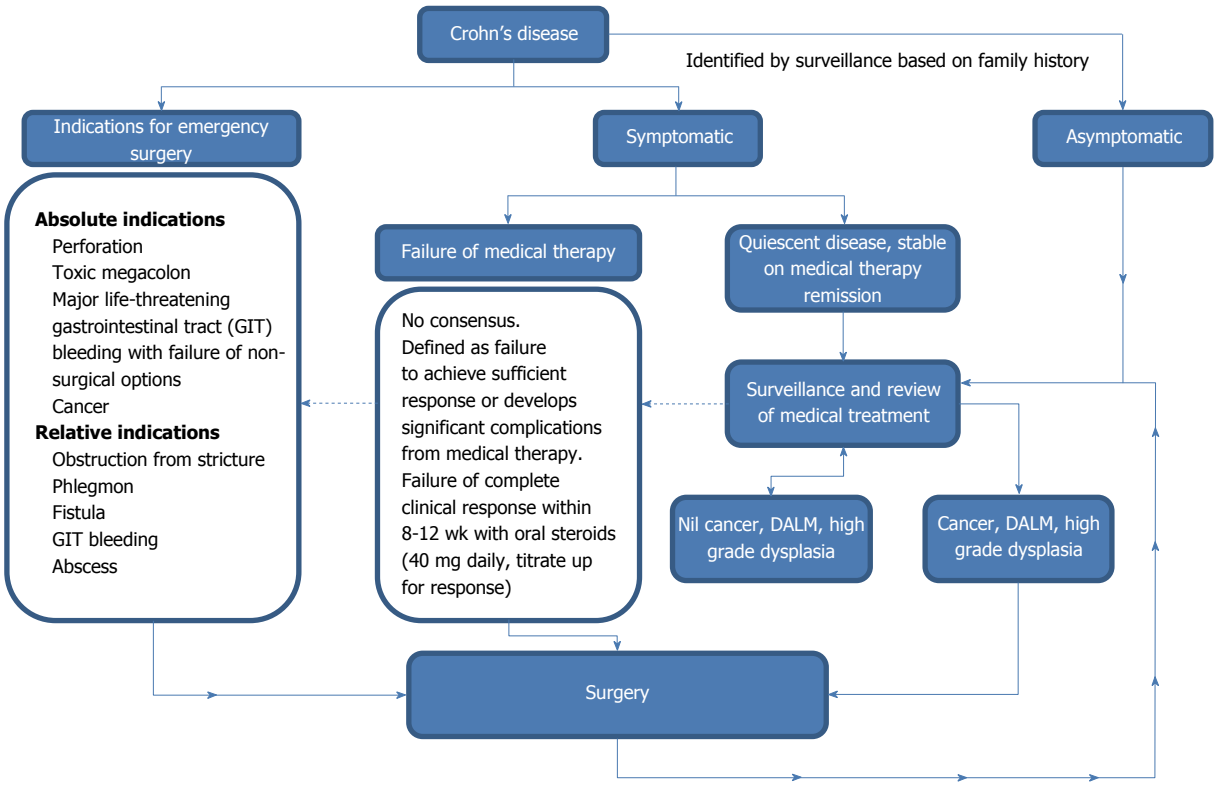

Figure 1 Indications for surgery in Crohn's disease. DALM: Dysplasia-associated lesion or mass.

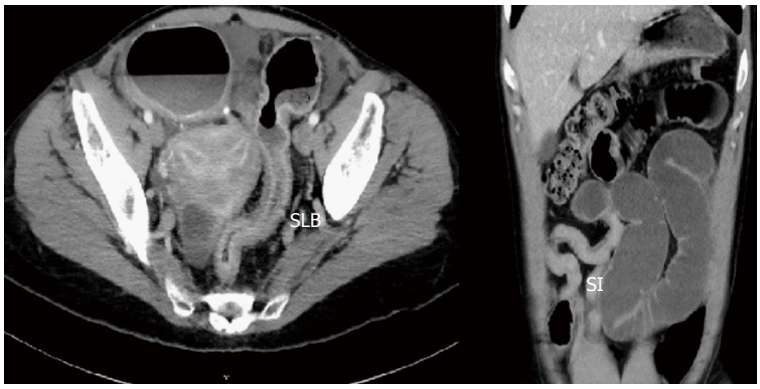

Figure 2 Stricturing disease. SLB: Strictured large bowel; SI: Strictured ileum.

strictures post-surgery for CD. Stricturing disease phenotype has often been associated with penetrating disease phenotype.

Intraabdominal phlegmon, abscesses and fistulae

Abscesses, phlegmons and fistulae in CD are associated with a penetrating phenotype. Younger patients with CD are more likely to have penetrating disease[10]. Intra-abdominal abscesses are common in CD, and may be associated with fistulae and/or intra-abdominal sepsis. Approximately one third of patients with CD develop intra-abdominal fistulae during their lifetime. Enteroenteric fistulae are the most common, followed by enterocutaneous and enterosigmoid fistulae[11]. Pregnancy is associated with higher rates of intestinal-genitourinary fistulas[12]. Occasionally, patients may present with phlegmon without abscess or fistula.

Perforation

Free perforation is the initial symptom of CD in 1%-3%[13] up to 30%[14]. Free perforation is the indication for surgery in 1%-16% of surgical intervention in CD[15]. The mean time from initial diagnosis to perforation is approximately 3 years, shorter in duration than development of fistulas, strictures and intra-abdominal abscesses[14]. Perforation may be from regional severe colitis or ileitis, or from toxic megacolon associated with fulminant colitis. A high index of suspicion is required as corticosteroids may

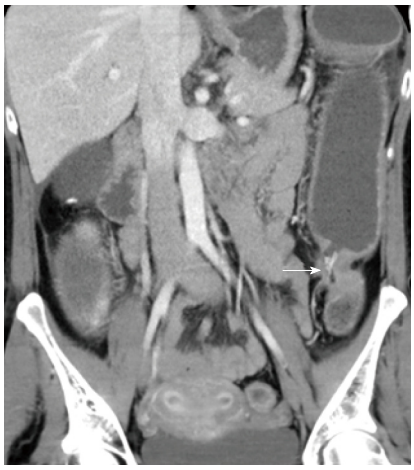

Figure 3 Large bowel stricture in Crohn's disease. Final pathology adenocarcinoma (arrow).

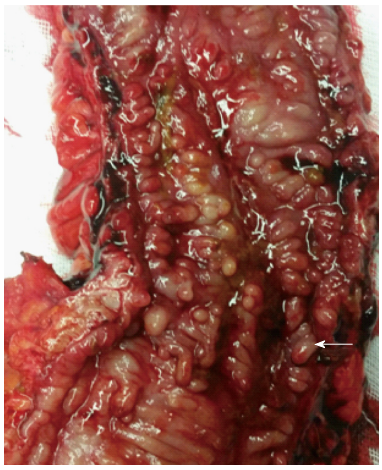

Figure 4 Pseudopolyps (arrow marks a pseudopolyp) in Crohn's disease.

mask the symptoms of perforation, leading to delayed management and increased mortality risk.

Failure of medical therapy

Failure of medical therapy is defined as failure to achieve sufficient response, development of significant complications from medical therapy, non-compliance with medical therapy, incompatibility with lifestyle or livelihood and steroid dependence with intolerability to other medications. There is no consensus as to what is sufficient response. A common definition is failure of complete clinical response with 8-12 wk of oral steroids and other agents. Approximately 20%-30% of CD patients do not respond to steroids, and up to 45% of CD patients will relapse on weaning of steroids[16]. Patients who have good response to medical therapy, and are able to tolerate immunosuppressants are usually recommended to remain on therapy for at least 3 to 4 years to reduce the likelihood of early relapse[17].

Oral prednisolone is usually started at 40 mg daily, and titrated up for clinical response for up to 12 wk[18] with commencement of immunomodulators or biological agents. As immunomodulators take up to 16 wk for response, some definitions of failed therapy suggest ongoing symptoms and signs despite medical therapy for up to 16 wk.

Major gastrointestinal bleeding

Severe gastrointestinal haemorrhage is rare[19]. Approximately 5% of CD patients will experience a major GIT bleed during their lifetime. It most commonly arises from ulcerated colonic areas, with colonic involvement more likely than small bowel involvement[20].

Toxic megacolon

In CD, the rate of toxic megacolon is approximately 2%[21]. Toxic megacolon is defined by total or segmental colonic distension and systemic toxicity[22]. Toxic megacolon may be associated with fulminant colitis and with co-infections such as clostridium difficile. Toxic megacolon may be associated with commencement of narcotics and anti-diarrhoeals used to manage bloody diarrhoea associated with severe colitis. For this reason, in general, anti-diarrhoeals should be avoided in management of colitis associated with CD.

Neoplasia and dysplasia

Cancer of the small bowel and colon is an absolute indication for surgery. The risk of cancer ranges from 1%-5% in CD[9,23,24], representing a 2-3 times increased risk of developing colorectal cancer and > 18 times increased risk of developing small bowel cancer when compared to the general population[25,26]. Patients may present with bowel obstruction (Figure 3), bleeding or non-specific gastrointestinal symptoms, or cancer may be detected during surveillance colonoscopy. Colorectal cancer in CD usually develops at an earlier age when compared to the baseline population[27]. For patients with CD colitis, the risk of developing cancer is 3%-4%[28]. Severe ileitis is associated with increased risk of small bowel cancer (approximately 2%). Dysplasia, earlier age of diagnosis of CD, extensive colitis or ileitis, length of disease > 10 years, presence of pseudopolyps (Figure 4), presence of anal fistula or other penetrating disease and primary sclerosing cholangitis is associated with increased risk of cancer in CD[28,29].

Growth retardation

One in 4 patients with CD are diagnosed before the age of 18 years old. CD has a bimodal distribution, with patients diagnosed during childhood and adolescence with a more severe phenotype. Growth retardation is associated with poor nutritional status associated with CD due to malabsorption and administration of corticosteroids[30].

SURGICAL OPTIONS IN SMALL BOWEL AND LARGE BOWEL CD

Stricture and obstruction

Stricturing CD, when associated with internal fistula, small bowel obstruction, bowel dilation > 3 cm or inflammatory phlegmon or abscess, are considered high risk strictures that usually require surgery[31]. Patients with fibrotic anastomotic strictures rather than de novo strictures are also more likely to require surgery[32].

Surgical options include resection of stricture with primary anastomosis or stricturoplasty. Stricturoplasty leads to higher surgical recurrence than resection[33].

Ileocolic

The most common surgery for stricturing disease in CD is ileocolic resection for ileocaecal or distal ileal disease[9]. Approximately 87% of patients with ileocaecal disease eventually require resection[34]. A significant proportion ileocolic resections for CD are performed in the emergency setting[35]. While there is no benefit from initiation of anti-TNF therapy prior to surgery for patients with severe ileocaecal CD[36], there is evidence that percutaneous drainage of abscess prior to surgery reduces the risk of anastomotic leak and rates of faecal diversion and this should be considered in stable patients without generalised peritonitis or clinical deterioration. If a CD patient is dependent on steroids and has a pre-operative abscess and requires immediate surgery, the risk of anastomotic complications is 40% and a stoma should be strongly considered[35].

Eighty percent of ileocolic resections are without clinical recurrence at 2 years[37] and the long term relapse rate is 36%[34]. Ileocolic resections are often complicated by chronic diarrhoea due to loss of ileo-caecal valve[38]. Novel methods of forming pseudo-valves, such as the nipple valve anastomosis technique have been reported[39], but there is no evidence to recommend routine formation of pseudovalves post ileocolic resection.

Approximately 30% of patients post ileocolic resection develop an anastomotic stricture. Dealing with an anastomotic stricture associated with recurrent CD is difficult and usually require resection if endoscopic balloon dilatation (EBD) fails. Stricturoplasties are associated with short term resolution of symptoms but a higher rate of recurrence than for resection[40], particularly in children and adolescents with CD.

Ileal

Stricturoplasties are considered a safe alternative to resection and are an important strategy to preserve bowel length.

The Heineke-Mikulicz and Finney techniques are the most common types of stricturoplasty procedures[41]. Other techniques of stricturoplasties such as the Michelassi technique and modified approaches have also been reviewed in meta-analyses, and are considered safe[42].

Colonic

For large bowel strictures not complicated by fulminant colitis, toxic megacolon or cancer, segmental resection and anastomosis with or without diversion for localised disease offers better quality of life, although it is associated with a higher risk of recurrence. Prabhakar *et al*[43] reported on 49 patients who had segmental colon resection without a permanent stoma for primary colonic CD followed up for over 10 years. Forty-five percent required no further treatment, 22% required medical therapy (only 8% required medical therapy for > 1 year) and only 33% required re-operation (10% multiple re-operations). A significant proportion remained stoma free, and of those who subsequently required a stoma, the average stoma-free interval was approximately 2 years.

A meta-analysis by Tekkis *et al*[44] reported that segmental colectomy with primary anastomosis was just as good as colectomy with ileorectal anastomosis for single segment colonic CD with no increase in recurrence. Recurrence rates only increased when segmental colectomies were performed for CD patients who had two or more colonic segments involved[44]. For this reason, segmental resections for localised colonic CD strictures or colitis is a reasonable option. Total colectomy is reserved for patients with multiple strictures, severe pancolitis, fulminant colitis, toxic megacolon or cancer.

Stricturoplasties may be performed for CD large bowel strictures with good functional outcomes[45]. It is not commonly performed as it is associated with increased recurrence rates when compared to resection[45]. There is however, no evidence that large bowel stricturoplasty increases the leak rate when compared to resection[45].

Non-surgical management of strictures

Not all stricturing disease requires surgery. The benefit of EBD is that 1 in 2 have sustained response and may avoid surgery[46], albeit requiring repeated EBD. Repeated interval stricture dilation has been successful in delaying the need for surgery without significant morbidity[47,48].

Despite short term success rates for EBD being favourable[49,50], there is an increased rate of re-intervention and complications with EBD. The long-term outcome after surgery is better than for EBD[51] and salvage surgery after failure of EBD is associated with higher risk of stoma, surgical site infections, reoperations and readmissions when compared with surgery first approach[52].

Stenting for CD strictures has been described in case reports with reasonable short term outcomes[53-55]. However, this is not commonly performed, and there is insufficient evidence to recommend this practice.

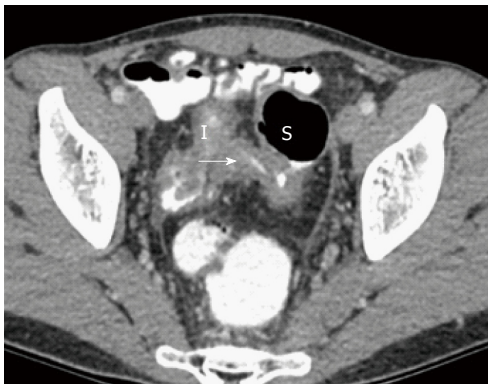

Figure 5 Ileosigmoid fistula with arrow marking contrast. Contrast flowing from ileum to sigmoid. I: Ileum; S: Sigmoid.

Intraabdominal phlegmon, abscesses and fistulae

Phlegmons: Abdominal phlegmons may be treated non-surgically with antibiotics and anti-TNF therapy without the need for surgery if the patient is stable with no evidence of generalised peritonitis or clinical deterioration. Successful management of phlegmon long term has been reported in the literature[56].

Intra-abdominal abscesses: The majority of intra-abdominal abscesses require definitive surgical management and intestinal resection[57]. Immediate surgical intervention should only be reserved for unstable patients with generalised peritonitis or clinical deterioration.

Drainage of abscess prior to surgery and delaying surgery reduces the risk of anastomotic complications. Emergency surgery for intra-abdominal abscess when patient is septic and on steroids is associated with high anastomotic leak rates[35].

There is, however, a high failure rate with percutaneous drainage alone. In paediatric patients with intraabdominal abscesses, over 60% of patients initially managed medically or treated with percutaneous drainage will require surgery within 1 year[57]. In adult CD, medical management and percutaneous drainage alone without removing the diseased intestine is associated with an unacceptably high failure rate[58], particularly for patients with larger abscesses or abscesses with a detectable fistula on imaging[59].

It is reasonable to consider non-surgical treatment of abdominal abscesses if there is no abscess or fistula on repeat imaging and there is no ongoing steroid requirement[60]. Initial management with non-surgical management may reduce the need for surgery in a small number of cases[61], although majority require surgery.

For larger or complex abscesses or when associated with fistula, planned bowel resection after initial management with percutaneous drainage of abscess, high dose steroids (up to 300 mg IV hydrocortisone per day) and IV antibiotics for at least 5 d increases the likelihood of primary anastomosis without the need for ileostomy, and avoids the morbidity of high failure rates with conservative management alone[58].

There is no evidence for surgical drainage of abscess without intestinal resection. This has no advantage over percutaneous drainage[62].

Intra-abdominal fistulae: Anti-TNF therapy heals about one third of fistulae. The rest require surgery. Traditionally, majority of intra-abdominal fistulae undergo intestinal resection and primary anastomosis[11]. The main caveats in CD fistula are large bowel fistulae are more likely to require surgery than small bowel fistulae[63] and colo-cutaneous and entero-cutaneous fistulae usually require surgical intervention[64], although in the era of anti-TNF therapy, medical management facilitates fistulae closure in up to one-third of patients[65]. (1) Small bowel fistulae: Long term remission may be achieved with medical therapy, such as ileovesical fistulae when there are no other CD complications. However, when small bowel CD fistulae are complicated by intraabdominal abscesses, small bowel obstruction or enterocutaneous fistulae, most cases usually still require surgery[63]. Surgery for enteric fistulae is associated with low rates of complications and recurrence[66]. Small bowel resection with primary anastomosis without diversion is usually associated with reasonable outcome. And (2) Large bowel fistulae: Surgery is nearly always indicated. A common large bowel fistula is the ileo-sigmoid fistula (Figure 5), which is a well-known manifestation of CD. These patients require ileocolic resection and either primary repair or segmental resection of the sigmoid, or a subtotal colectomy. In a study of ileocolic resection and primary repair of sigmoid *vs* ileocolic resection and segmental resection of sigmoid *vs* subtotal colectomy, ileocolic resection with primary repair or segmental sigmoid resection was shown to have comparable morbidity[67]. However, there have been reports of increased rates of postoperative sepsis with sigmoidectomy when compared with primary repair of sigmoid[68]. Subtotal colectomy is not required unless there is moderate-severe pancolitis.

Perforation

Perforation is an absolute indication for surgery. Surgical management depends on the site of perforation. Simple suture closure of the perforation is associated with a high mortality rate, and intestinal resection of the perforated viscus is warranted. If there is faecal contamination, abscess or the patient is on high dose steroids, then primary anastomosis is associated with significant risk of anastomotic complications. Patients on corticosteroid dose equivalent to 20 mg of prednisolone or greater have anastomotic complication rates of up to 20%[69]. If perforation is associated with severe ileitis, a temporary abcarian stoma may be safer than primary anastomosis. If

perforation is associated with severe CD colitis, a segmental resection, subtotal or total colectomy and end ileostomy may be performed with an exteriorised mucus fistula or stump placed intraperitoneally or subcutaneously. In considering whether to provide the patient with a stoma in the setting of perforated CD, high degree of contamination, low albumin[70] and presence of intra-abdominal abscess[35] should be considerations towards faecal diversion.

Failure of medical therapy

CD patients who continue to have symptoms of abdominal pain, severe diarrhoea, bleeding, obstructive symptoms, weight loss, malabsorption, dehydration or signs of systemic toxicity despite 12-16 wk of medical therapy including steroids and first and second line immunomodulators and biological agents require surgery. Surgical options depend on the phenotype of CD.

In patients with severe CD requiring emergency admission to the hospital, failure to improve with steroids over a 72 h period may require escalation to surgery.

Not all patients should wait 12-16 wk before escalating to surgery, particularly patients with severe stricturing ileocolic disease which usually fails conservative management[36], or patients with risk factors for further medical therapy. These patients should have surgery earlier than 12-16 wk if not responding early to medical therapy.

Major gastrointestinal bleeding

The majority of bleeding resolves spontaneously or with medical management - conservative measures are successful in 80% of cases. This includes blood transfusions, supportive measures, corticosteroids, cyclosporins and biological agents. However, 40% of patients with massive bleeding experience severe re-bleeding episodes. Medications such as infliximab are useful in combating the long term risk of bleeding recurrences[71]. To control acute massive bleeding, interventions such as angio-embolisation and endoscopic treatment should be attempted, but often fail. This is because of difficulties in identifying a precise bleeding point both angiographically and endoscopically as bleeding usually occurs in multiple inflamed areas[20]. From a therapeutic point of view, endoscopic or radiological haemostasis has a low rate of success[19].

Surgery is reserved to salvage patients with intractable bleeding who clinically deteriorate or become haemodynamically unstable[72]. A total colectomy is indicated in life-threatening cases refractory to non-surgical management. Surgery usually is effective with a low risk of rebleeding[20], however is associated with high morbidity.

Toxic megacolon

A diameter of the transverse colon of > 5.5 cm with systemic toxicity and abdominal pain in a CD patient is an indication for surgery[22].

Steroids and biological agents are often used but medical management including bowel rest and total parenteral nutrition often fail.

Subtotal colectomy, end ileostomy and subcutaneous placement of sigmoid stump or exteriorisation of the stump is associated with lower mortality than total colectomy in the management of toxic megacolon[73]. The decision to operate is based on clinical acumen as emergency surgery is guided by clinical signs of impending perforation rather than an exact diameter of colon demonstrated on CT or X-ray.

Dealing with the retained rectum or rectosigmoid is an important consideration after subtotal colectomy. There is no evidence that a second stoma or mucus fistula improves outcome. Subcutaneous placement of the stump has the lowest reported morbidity. Intraperitoneal placement is associated with a significantly higher risk of pelvic sepsis from stump blowout[74].

Cancer

The recent practice guidelines by Strong *et al*[15] on surgical management of CD provided strong recommendations for total proctocolectomy for patients with cancer, DALM, high grade dysplasia or multifocal low grade dysplasia. The rationale for total proctocolectomy is the high rate of metachronous cancers seen following segmental or subtotal colectomies associated with CD[75] - up to 40% for segmental resection, and 35% for subtotal colectomy. The mean time for development of metachronous cancer is approximately 7 years from initial surgery[75]. While the current guidelines recommend total proctocolectomy for CD associated colon cancer, a meta-analysis has shown increased risk of colon cancer but not rectal cancer in CD[26] and a total colectomy with surveillance of the remaining rectum is a reasonable option.

Dysplasia

The risk of cancer with high grade dysplasia is > 70%, and with low grade dysplasia about 30%-40%[76]. Approximately 50% of cancers in CD is associated with dysplasia[77].

Dysplasia is usually multifocal in CD colitis, and for this reason, segmental or subtotal colectomy for dysplasia is associated with high risk of developing subsequent malignancies. For patients assessed to be fit for surgery, total colectomy or proctocolectomy is recommended. High risk surgical candidates should have close endoscopic surveillance if the risks of surgery for dysplasia outweigh the benefits[76].

Dysplasia surveillance: CD colitis is associated with increased risk of cancer. Yearly colonoscopy is recommended for high risk CD patients who have one or more high risk factors including moderate to severe active inflammation, colonic stricture or dysplasia in

the past 5 years, primary sclerosing cholangitis or family history of colorectal cancer < 50 years old. 3-yearly colonoscopy is recommended for intermediate risk CD patients including mild active colitis, inflammatory polyps or family history of colrectal cancer > 50 years old. Patients are low risk if there is no active inflammation (even with extensive colitis < 50% of colon), and these patients require 5-yearly colonoscopy[78].

Growth retardation

Paediatric CD may be complicated by growth retardation. Well-timed surgery for CD before puberty begins can help to control disease activity and improve nutritional status and reduce corticosteroid requirement[30]. However, early surgical recurrences are common due to more severe CD phenotype in younger patients, and this may limit the benefit of surgery[79].

INDICATIONS FOR SURGERY IN PERIANAL CD

Perianal pathology occurs in 40%-80% of patients with CD[80]. Colonic and rectal CD phenotypes are associated with increased risk of perianal disease[81,82]. Anal fistulae in CD may be secondary to CD or associated with cryptoglandular origin. Anal cancers are rare in CD. There is not a significant increase in incidence of anal cancers in CD compared to the normal population.

SURGICAL OPTIONS FOR PERIANAL CD

Perianal fistulae and abscesses

Perianal disease causes significant impairment in the quality of life for CD patients[83]. 20% of CD patients require perianal surgical intervention at some stage[84] although the rate of surgery for perianal disease in CD is falling with increasing use of thiopurine and infliximab therapy[85].

The management of perianal abscesses in CD is the same as for the normal population - incision and drainage of abscess. Perianal abscesses may often be associated with complex fistulating disease or may be crypto-glandular. Endoanal ultrasound and magnetic resonance imaging (MRI) may be useful to evaluate complex perianal disease.

For perianal fistulae, medical therapy is the mainstay of treatment. Surgery is reserved for patients who develop abscesses or sepsis. Anti-TNF therapy has been shown to be an effective treatment for closure of perianal fistulizing CD[65]. Infliximab or adalimumab step up therapy for CD guided by assessment of disease severity by anal ultrasound is associated with a high rate of fistula closure[86].

Low CD perianal fistulae may be treated by fistulotomy. Complex or high CD fistulae should be managed with placement of long-term setons. There is limited evidence for advancement flap closures, debridement, fistula plug and fibrin glue. The current gold standard is long-term setons with infliximab therapy. Closure rates of up to 60% for perianal CD fistulae have been reported,although results have been mixed[87]. If the patient is asymptomatic, however, there is no need for surgery or setons.

There is a very limited role for faecal diversion. As biological drugs may induce full regression in 80% of cases of anorectal disease[88], diversional stoma for perianal disease should be reserved for difficult cases refractory to medical therapy and drainage. There is a high likelihood that "temporary" faecal diversion to manage severe perianal CD is usually permanent[89,90]. Sauk *et al*[90] reported 49 patients who underwent faecal diversion for severe CD perianal disease of which 15/49 (30.6%) had reversal of stoma but ten of these (66.7%) required re-diversion of the faecal stream. Of the five patients who maintained intestinal continuity, three required further surgical interventions to control sepsis. The likelihood of restoration of intestinal continuity post faecal diversion is less than 20%[89]. In CD, perianal disease remains the most common reason for stoma[9].

Only patients with very severe anorectal involvement with CD, including anovaginal or rectovaginal fistulae, should have total proctocolectomy as anorectal CD usually responds well to biological agents with full regression of disease.

Anal cancer

Anal cancers are not common in CD and are difficult to diagnose. Only 61 cases of carcinomas arising in perineal fistulas associated with CD was reported in a systematic review in 2009[91].

In the majority of reported case, biopsies were only proven in 20% of cases[91]. A high index of suspicion for malignancy is required as biopsies of malignancy have a high false negative rate, with malignant cells deep within fistulae. In CD anal cancers, there is similar incidence of adenocarcinomas and squamous cell cancers.

The treatment modalities for anal cancers associated with CD is the same as with anal cancers in the normal population.

Anal fissures and tags

Anal fissures and bulky skin tags are common in CD. Avoid extensive surgery due to poor wound healing for skin tags and fissures. In the management of fissures, avoid lateral sphincterotomy. Management with lifestyle changes, botox and immunomodulators is preferable to surgery.

TECHNICAL CONSIDERATIONS IN SURGERY FOR CD

Minimally invasive surgery

Laparoscopic colectomy is safe in the management

of CD[92,93] and has short term benefits[94-96] including reduced blood loss, decreased rates of ileus and shorter length of stay[97,98] as well as decreased incisional hernia rates[95]. The main benefits of laparoscopic surgery are short term. Reoperation rates, endoscopic and radiologic recurrence are similar between both open and laparoscopic groups[99]. A potential long term benefit of laparoscopic surgery may include lower incidence of adhesional bowel obstructions[100].

Importantly, laparoscopic colectomy does not add to morbidity[67] and may reduce the risk of postoperative enteric fistula when compared with open surgery[101]. Laparoscopic surgery may be used successfully in complex CD[102] and recurrent CD[103]. While laparoscopic surgery has also been shown to be safe in patients with previous midline laparotomy for intestinal resection for CD, no significant advantage, except reduced wound infection rates, has been demonstrated[104].

The conversion rate for laparoscopic surgery in the setting of CD has been reported between 8.5%[103] to 13.4%[105]. The predictors for conversion include complex fistulating disease and the need to carry out multiple stricturoplasties[103].

Single incision laparoscopic surgery in CD has been shown to be safe, with results comparable with traditional laparoscopic procedures[106-108].

Anastomosis

End-to-end, side-to-side and end-to-side anastomoses have comparable recurrence rates despite early reports of differences, and all configurations are reasonable in the setting of CD[15].

Specifically for ileocolic anastomosis, however, a recent meta-analysis showed that a side-to-side stapled anastomosis is associated with decreased rates of anastomotic leak and decreased surgical recurrence[109].

The risk of anastomotic leak in CD varies from 3%[110] to 20%[69]. Leaks are usually diagnosed late in the postoperative period[111]. Colo-colonic anastomosis are associated with a higher risk of anastomotic complications when compared to entero-colic or enteroenteric anastomosis[69].

Major risk factors of anastomotic leak include low albumin[70], intra-abdominal abscess[35] corticosteroids[35], particularly patients on corticosteroid steroid dose equivalent to 20 mg of prednisolone or greater, with anastomotic complications reported between 20%[69].

Biologic treatment with infliximab or other anti-TNF therapy and immunomodulators pre-operatively have not been shown to have an increased risk of anastomotic leak[69,112], and should not be a contraindication to primary anastomosis.

Pouch

Pouches are not generally recommended for CD. The ileal pouch anal anastomosis (IPAA) is associated with high failure rates and poor functional outcomes[113], particularly for CD with NOD2 mutation which is associated with severe pouchitis[114,115]. Inflammation in the pouch is significantly higher for CD than for UC[116]. However, most cases of pouchitis can be managed with antibiotics, and those that are antibiotic resistant may be treated successfully with thiopurines alone[117]. Stricturing disease of the pouch also respond well to thiopurines but fistulising disease does not respond well to medical therapy and even with step up therapy to infliximab, the stoma rates are high[117].

The risk of malignancy within the IPAA associated with CD is small, with only a handful of case reports.

CONCLUSION

The management strategies of CD are largely dependent on phenotypic classification and indication. Advances in surgical management has reduced perioperative mortality rates to < 1%[118], with minimally invasive surgery shown to be safe in CD. However, unfortunately, improvements in surgical techniques have only been accompanied by modest improvements in surgical rates, recurrences and overall mortality.

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P- Reviewer: Galanakis CG, Guerra F **S- Editor**: Qi Y **L- Editor**: A
E- Editor: Wang CH

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DOI: 10.3748/wjg.v22.i40.8905

World J Gastroenterol 2016 October 28; 22(40): 8905-8909
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

MINIREVIEWS

Effects of a high fat diet on intestinal microbiota and gastrointestinal diseases

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Author contributions: The authors equally contributed to this work.

Conflict-of-interest statement: There are no potential conflicts of interest and no financial support was given.

Manuscript source: Invited manuscript

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Received: July 1, 2016
Peer-review started: July 4, 2016
First decision: August 2, 2016
Revised: August 15, 2016
Accepted: September 14, 2016
Article in press: September 14, 2016
Published online: October 28, 2016

Abstract

Along with the rapid development of society, lifestyles and diets have gradually changed. Due to overwhelming material abundance, high fat, high sugar and high protein diets are common. Numerous studies have determined that diet and its impact on gut microbiota are closely related to obesity and metabolic diseases. Different dietary components affect gut microbiota, thus impacting gastrointestinal disease occurrence and development. A large number of related studies are progressing rapidly. Gut microbiota may be an important intermediate link, causing gastrointestinal diseases under the influence of changes in diet and genetic predisposition. To promote healthy gut microbiota and to prevent and cure gastrointestinal diseases, diets should be improved and supplemented with probiotics.

Key words: Intestinal microbiota; Gastrointestinal diseases; High fat diet

Core tip: Along with the rapid development of society, lifestyles and diets have gradually changed. Due to overwhelming material abundance, high fat, high sugar and high protein diets are common. Numerous studies have determined that diet and its impact on gut microbiota are closely related to obesity and metabolic diseases. Different dietary components affect gut microbiota, thus impacting gastrointestinal disease occurrence and development. A large number of related studies are progressing rapidly. In this review, we summarize the relationship between a high fat diet, gut microbiota and gastrointestinal diseases.

Zhang M, Yang XJ. Effects of a high fat diet on intestinal microbiota and gastrointestinal diseases. *World J Gastroenterol* 2016; 22(40): 8905-8909 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i40/8905.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i40.8905

INTRODUCTION

Along with the rapid development of society, lifestyles and diets have gradually changed. Due to overwhelming material abundance, high fat, high sugar and high protein diets are common. Numerous studies have determined that diet and its impact on gut microbiota are closely related to obesity and metabolic diseases[1]. Different dietary components affect gut microbiota, thus impacting gastrointestinal disease occurrence and development. A large number of related studies are progressing rapidly. In this review, we summarize the relationship between a high fat diet, gut microbiota and gastrointestinal diseases.

BASIC COMPOSITION OF INTESTINAL MICROBIOTA

The intestinal tract is the primary site of bacterial colonization in the human body. These complex and diverse bacteria form the gut flora. There are more than 1000 bacterial species in the human gut and this number can reach as high as 1×10^8 species. The intestinal flora is primarily composed of anaerobes, facultative anaerobes and aerobes. Anaerobes comprise more than 99% of gut microbes. The intestinal flora of the human body primarily includes Firmicutes, Bacteroidetes, Actinomycetes, Proteobacteria, Verrucomicrobia and Archaebacteria. More than 90% are Firmicutes or Bacteroidetes. The Firmicutes, Bacteroidetes, Proteobacteria and Actinomycetes comprise 64%, 23%, 8% and 3% of the gut microbiota, respectively[2]. The intestinal flora of the human body is established in infancy and gradually stabilizes with age. By approximately 2 years of age, it is similar to the adult intestinal flora[3]. The intestinal flora composition differs by age group. The proportion of Firmicutes and Bacteroidetes in infants, adults and the elderly is 0.4, 0.9 and 0.6, respectively[4].

EFFECT OF A HIGH FAT DIET ON INTESTINAL MICROBIOTA

Diet is an important factor determining intestinal flora composition. It plays a critical role in the colonization, maturation and stability of the intestinal flora. Both animal and human experiments have demonstrated that dietary changes can rapidly affect intestinal flora structure. Within 4 d of eating a specific dietary component, the human intestinal flora composition will change significantly[1,5].

Animal experiments have indicated that dietary structure affects intestinal flora. The proportion of Bacteroidetes decreased and the proportion of Firmicutes increased, which increased the proportion of Mollicutes in the intestinal tracts of mice fed a high fat and high sugar diet compared with mice fed a low fat and high sugar diet[6]. Intestinal flora diversity is reduced in mice fed a high fat and high sugar diet. However, control diet consumption gradually reversed these changes. Furthermore, one study investigated varying proportions of dietary fatty acids in mice for 8 wk. A diet high in saturated fatty acids led to an increased proportion of intestinal Firmicutes and decreased intestinal flora diversity[7]. This study suggests that dietary fats and saturated fatty acid intake may affect intestinal flora composition. One study found that converting a low sugar, low fat diet to a high sugar, high fat diet caused a rapid decline in the number of Bacteroidetes in the intestines[8]. Another study also suggested that the number of *Bacillus bifidus* was reduced in mice fed a high fat diet[9]. Animal studies have demonstrated a significant reduction in the number of lactic acid bacteria, *Bacillus bifidus* and *Enterococcus* in the intestinal tract of the group fed a high fat diet. Furthermore, the phylum Bacteroidetes displayed a decreasing trend, while the *Bacillus fusiformis* displayed an increasing trend[10,11].

Human experiments have also demonstrated that dietary composition affects intestinal flora. Compared with Italian children who consume a large amount of plant protein, fat, sugar and starch, the proportion of Bacteroidetes in the intestinal flora of African children was high, while the proportion of Firmicutes was low[12] (Table 1).

RELATIONSHIP BETWEEN INTESTINAL MICROBIOTA AND GASTROINTESTINAL DISEASES

The composition and proportion of gut microbiota are closely related to human health. Upsetting the gut microbiota equilibrium can cause enteric dysbacteriosis and a variety of gastrointestinal and systemic diseases[13].

Intestinal microbiota and inflammatory bowel disease

Inflammatory bowel disease (IBD) comprises a group of inflammatory conditions of the colon and small intestine, including Crohn's disease (CD) and ulcerative colitis (UC), the cause and pathogeny of which are not completely understood. Gut microbiota are closely related to IBD occurrence and development. Although the specific bacteria involved in IBD have not been identified, the gut microbiota in patients with IBD differs from those of healthy individuals. One study[14] determined that the total number of mucosa-associated bacteria in the IBD group was higher than that in the control group. In the CD group, *Streptococcus* was dominant in the inflammatory mucosal region, while in the UC group, lactic acid *Bacillus* was dominant. Studies have demonstrated that the number of *Faecalibacterium prausnitzii* decreased in patients with CD[15]. Their secretory products have immune regulatory activity *in vitro*[16]. IBD pathogenesis includes intestinal flora imbalance,

Table 1 Effect of a high fat diet on intestinal microbiota

Diet	Intestinal flora	Animal experiments	Human experiments
High fat diet	Bacteroidetes	Decreased	Decreased
	Firmicutes	Increased	Increased
Low fat diet	Bacteroidetes	Increased	Increased
	Firmicutes	Decreased	Decreased

increased pathogenic bacteria, toxin damage to the intestinal epithelium, immune function abnormalities and immune tolerance imbalance. Intestinal bacteria can induce epithelial endoplasmic reticulum stress, leading to intestinal mucosal barrier damage and increased intestinal permeability. Probiotic supplements in patients with IBD can effectively alleviate symptoms and delay disease progress[17,18].

Intestinal microbiota and irritable bowel syndrome

Irritable bowel syndrome (IBS), affecting approximately 5%-25% of the population, comprises a group of symptoms, including abdominal pain and changes in bowel movement patterns, without any evidence of underlying damage. The mechanisms of IBS are unclear. One study found that 3%-36% of intestinal infections can cause persistent symptoms of IBS, which suggests that gut microbiota play an important role in IBS onset[19]. Intestinal flora may affect gastrointestinal motility, visceral sensitivity, the inflammatory response and the brain-gut axis, which leads to IBS. A number of studies have confirmed that the intestinal flora of patients with IBS differs from that of healthy individuals[20,21]. At present, however, intestinal flora composition results in patients with IBS have been inconsistent and some have been contradictory. These inconsistencies may be owing to differences in specimen collection, molecular detection methods or definitions of IBS[22]. The majority of studies have found that the Bacteroidetes are reduced, while the Firmicutes are increased in the intestinal flora of patients with IBS. However, it is not yet determined whether the changes in intestinal flora directly cause or are secondary to IBS. In the future, treatment of the intestinal flora imbalance may become an option for patients with IBS[23].

Intestinal microbiota and tumors

Colorectal cancer is a common gastrointestinal tumor, the incidence and mortality rates of which are increasing each year. Most colorectal cancers are due to old age, lifestyle factors and underlying genetic disorders. Additionally, changes in the gut microbiota are closely related to colorectal cancer occurrence and development[24]. Many studies have detected imbalances in the gut microbiota of patients with colorectal cancer, while those of healthy individuals are in equilibrium. Furthermore, some reports have suggested that changes in the gut microbiota can cause cancer directly. However, it is unclear which species of bacteria play a primary role in causing cancer[25-27]. There are two theories regarding the pathogenesis of colorectal cancer associated with intestinal flora. First, some intestinal bacteria may either directly or indirectly affect intestinal epithelial cells, causing genetic mutations. These bacteria are defined as "Alpha-bugs"[28]. Their direct effects include secreting toxic proteins and the indirect effects include changes in intestinal flora that are more likely to cause mucosal immune responses and changes in colonic epithelial cells. When gene mutations accumulate, it can lead to colorectal cancer. The second theory is named the "driver-passenger" model[29]. Following colorectal cancer incidence, primary pathogens (defined as "drivers") are replaced by opportunistic pathogens (defined as "passengers"), which are more viable in the intestinal tumor microenvironment. Possible mechanisms of intestinal flora-induced colon cancer are summarized as follows: (1) the carcinogen precursor is absorbed by the stomach, then secreted into the intestinal cavity by the liver and the active ingredient is released by intestinal flora activity; (2) the carcinogen precursor in food is released by intestinal flora activity; and (3) metabolites produced by intestinal flora induce carcinogenic effects. Many studies have explored the role of probiotics in colon cancer prevention[30]; however, there is not yet a consensus.

Intestinal microbiota and liver disease

The intestinal blood flows through the portal vein system to return to the liver. The liver affects intestinal function by secreting bile into the enterohepatic circulation. The physiological link between the two organ systems is called the "intestine-liver axis". Studies have indicated that changes in intestinal flora play an important role in liver disease incidence and progression[31]. Intestinal probiotics can improve liver disease and are now widely used in its clinical treatment[32]. Nonalcoholic fatty liver disease (NFALD) is one of the most rapidly growing chronic liver diseases. A number of studies have indicated that intestinal flora play an important role in NFALD development[33]. Bacterial overgrowth and intestinal permeability are the primary mechanisms underlying endotoxemia and inflammatory reaction-initiated liver disease. One study confirmed the relationship between intestinal bacterial overgrowth and NFALD[34]. In another study, the relationship between intestinal permeability and NFALD was demonstrated in animal experiments[35]. Alcoholic fatty liver was also associated with gut-derived endotoxemia. Specifically, ethanol intake in the intestinal tract may cause intestinal mucosal injury and intestinal flora disorder, resulting in increased endotoxin-induced intestinal epithelial permeability, bacterial translocation and endotoxemia[36]. The intestinal flora in patients with liver cirrhosis is dramatically disordered. One study demonstrated

a significant decrease in *Bacillus bifidus* and lactic acid *Bacillus* in the intestinal tract of patients with liver cirrhosis, suggesting the possibility of intestinal bacterial translocation and increased infection[37]. The occurrence of primary hepatocellular carcinoma is also associated with intestinal flora imbalance[38].

CONCLUSION

In summary, gut microbiota may be an important intermediate link, causing gastrointestinal diseases under the influence of changes in diet and genetic predisposition. A diet that is high in fat, especially high in saturated and trans fat, is closely related to obesity, metabolic syndrome and gastrointestinal diseases; polyunsaturated fats such as omega-3, omega-6 and omega-9 in right proportions are suggested as substitutes. To promote healthy gut microbiota and to prevent and cure gastrointestinal diseases, diets should be improved with low fat, low sugar, high fruit and vegetable intake and complex fibers and supplemented with probiotics or increased fermented dairy product consumption, such as yogurt and buttermilk. It is essential for patients with GI diseases to not only change their dietary composition, but also to establish a healthy eating habit and pattern to promote healthy microbiota as well as to alleviate disease-associated syndromes. Maintenance of normal gut microbiota may be a potentially key means of preventing GI diseases in the future.

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P- Reviewer: Czubkowski P, Gobejishvili L **S- Editor**: Qi Y
L- Editor: Roemmele A **E- Editor**: Zhang FF

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DOI: 10.3748/wjg.v22.i40.8910

World J Gastroenterol 2016 October 28; 22(40): 8910-8917
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

ORIGINAL ARTICLE

Basic Study

Polymorphisms and resistance mutations of hepatitis C virus on sequences in the European hepatitis C virus database

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Author contributions: Kliemann DA designed this study, acquired and analyzed data; Kliemann DA and Wood C interpreted the data; Tovo CV, da Veiga ABG, de Mattos AA and Wood C contributed to writing of article, editing, reviewing and final approval of the article.

Institutional review board statement: The research protocol was approved by the Research Ethics Committee at Hospital Nossa Senhora da Conceição 12089/2012, approval report 12/2012.

Conflict-of-interest statement: The authors declare that no conflict of interest exists.

Data sharing statement: All available data can be obtained by contacting the corresponding author.

Manuscript source: Invited manuscript

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Received: June 19, 2016
Peer-review started: June 21, 2016
First decision: August 8, 2016
Revised: September 12, 2016
Accepted: September 28, 2016
Article in press: September 28, 2016
Published online: October 28, 2016

Abstract

AIM

To evaluate the occurrence of resistant mutations in treatment-naïve hepatitis C virus (HCV) sequences deposited in the European hepatitis C virus database (euHCVdb).

METHODS

The sequences were downloaded from the euHCVdb (https://euhcvdb.ibcp.fr/euHCVdb/). The search was performed for full-length NS3 protease, NS5A and NS5B polymerase sequences of HCV, separated by genotypes 1a, 1b, 2a, 2b and 3a, and resulted in 798 NS3, 708 NS5A and 535 NS5B sequences from HCV genotypes

1a, 1b, 2a, 2b and 3a, after the exclusion of sequences containing errors and/or gaps or incomplete sequences, and sequences from patients previously treated with direct antiviral agents (DAA). The sequence alignment was performed with MEGA 6.06 MAC and the resulting protein sequences were then analyzed using the BioEdit 7.2.5. for mutations associated with resistance. Only positions that have been described as being associated with failure in treatment in *in vivo* studies, and/or as conferring a more than 2-fold change in replication in comparison to the wildtype reference strain in *in vitro* phenotypic assays were included in the analysis.

RESULTS

The Q80K variant in the *NS3* gene was the most prevalent mutation, being found in 44.66% of subtype 1a and 0.25% of subtype 1b. Other frequent mutations observed in more than 2% of the NS3 sequences were: I170V (3.21%) in genotype 1a, and Y56F (15.93%), V132I (23.28%) and I170V (65.20%) in genotype 1b. For the NS5A, 2.21% of the genotype 1a sequences have the P58S mutation, 5.95% of genotype 1b sequences have the R30Q mutation, 15.79% of subtypes 2a sequences have the Q30R mutation, 23.08% of subtype 2b sequences have a L31M mutation, and in subtype 3a sequences, 23.08% have the M31L resistant variants. For the NS5B, the V321L RAV was identified in 0.60% of genotype 1a and in 0.32% of genotype 1b sequences, and the N142T variant was observed in 0.32% of subtype 1b sequences. The C316Y, S556G, D559N RAV were identified in 0.33%, 7.82% and 0.32% of genotype 1b sequences, respectively, and were not observed in other genotypes.

CONCLUSION

HCV mutants resistant to DAAs are found in low frequency, nevertheless they could be selected and therapy could fail due resistance substitutions in HCV genome.

Key words: Hepatitis C virus resistance; Quasispecies; Direct antiviral agents; Polymorphisms; Drug resistance

Core tip: Chronic hepatitis C virus (HCV) infection is a significant cause of morbidity and mortality. The main therapeutic targets are the NS3/4A protease, NS5B polymerase, and NS5A replication complex. Pre-existence of resistance associated variants to direct antiviral agents (DAA) reduces sustained virologic response rates. Despite the low frequency of mutations, this resistant population is likely to be selected in patients undergoing therapy with DAA. Even though HCV variants resistant to DAA targeting one viral protein remain susceptible to DAA targeting another viral protein, combination therapy could fail due to selection of HCV with resistance substitutions in multiple targets.

Kliemann DA, Tovo CV, da Veiga ABG, de Mattos AA, Wood C. Polymorphisms and resistance mutations of hepatitis C virus on sequences in the European hepatitis C virus database. *World J Gastroenterol* 2016; 22(40): 8910-8917 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i40/8910.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i40.8910

INTRODUCTION

Chronic hepatitis C virus (HCV) infection affects around 180 million people worldwide and is a significant cause of liver-related morbidity and mortality[1] until recently. Interferon-α in combination with Ribavirin was the mainstream treatment regimen but eligibility and safety of the interferon-based therapies were low, and consequently the overall effectiveness of the treatment was very limited. Fortunately, the development of new direct-acting antiviral (DAA) drugs against HCV has progressed significantly and resulted in oral interferon-free therapies[2].

The three main therapeutic targets for HCV infection are the NS3/4A protease, the NS5B polymerase, and the NS5A replication complex. The first series of interferon-free regimens, including combinations of simeprevir (SMV), sofosbuvir (SOF), paritaprevir, daclatasvir, ledipasvir (LDV), ombitasvir (OMV), dasabuvir (DSV), grazoprevir (GZR) and elbasvir have already been approved and recommended by the European Association for the Study of the Liver (EASL) and by the American Association for the Study of Liver Diseases (AASLD)[3,4].

HCV variants infecting the human population show extreme genetic diversity, which is partly explained by the long evolutionary association between the virus and its human host. HCV exists in the host as a swarm of related quasispecies. This diversity is a result of the error-prone viral polymerase combined with rapid viral replication, which, in turn, enables the virus to rapidly overcome the host immune responses and to become resistant to antiviral drugs[5]. The selection of resistance-associated amino-acid variants (RAV) from HCV quasispecies is dependent on drug-, host- and virus-related factors. The potency of the drug itself is primarily influenced by viral susceptibility, by previous exposure to the drug and by the genetic barrier to resistance. The ability of a RAV to persist and to induce treatment failure (relapse, non-response or viral breakthrough) is related to its fitness or its replication capacity as compared to the wild-type virus[6,7].

Resistance to DAAs is driven by the selection of mutations at different positions in the NS3 protease, NS5B polymerase and NS5A protein[8,9]. Each compound or drug family induces a specific mutation profile that may be characteristic of the viral genotype/subtype. Furthermore, each class of DAA is characterized by a difference in the genetic barrier to resistance. Even though the specific resistance mutation for each individual agent in the drug class differs, there is a

great concern about the possibility of cross-resistance between compounds in the same inhibitor class, especially for the NS3 protease and NS5A inhibitors[10].

The ability to detect RAV depends primarily on the different types of the sequencing technologies used, including population-based sequencing, clonal sequencing and deep sequencing. The sensitivities for detection by these three approaches were reported to be approximately 25%, 5% and 0.5%, respectively, and the presence of viral mutants below the detection levels might be missed[11]. For HCV the frequency of routine testing of drug resistance prior to the use of the new treatment regimens is not known. Some guidelines[4] suggest that routine monitoring for HCV drug resistance-associated variants during therapy should not be recommended and there is no consensus on the utility of pre-treatment resistance testing.

Currently, there is a large number of HCV sequences available on public databanks, however they have not been analyzed to correlate HCV genotypes and viral genomic characteristics with drug resistance phenotypes[11]. Three HCV databases are currently available to provide insight into the basic biology, immunology, and evolution of the virus: the Japanese database (http://s2as02.genes.nig.ac.jp), the European database (http://euhcvdb.ibcp.fr) and the American database (Los Alamos National Laboratory) (http://hcv.lanl.gov).

The objective of this study is to evaluate the occurrence of polymorphisms and resistant mutations in the NS3, NS5A and NS5B regions in treatment-naïve HCV sequences deposited in the European hepatitis C virus database (euHCVdb). This analysis will provide insights into the levels of circulating drug resistance, which may affect the success of the therapeutic regimens.

MATERIALS AND METHODS

HCV database

The sequences were downloaded from the euHCVdb (https://euhcvdb.ibcp.fr/euHCVdb/). This bank provides important data about the HCV sequences (*e.g.* genotype, genomic region, viral proteins and their functions, known 3-dimensional structures) and ensures consistency of the annotations, which enables reliable keyword queries. Users can extract subsets of sequences obtained by Sanger sequencing matching particular criteria or enter their own sequences and analyze them with various bioinformatics programs available on the same server. The euHCVdb is mainly oriented towards protein sequence, structure and function analyses and structural biology of HCV, and is re-built every month from an up-to-date database by an automated process[12].

The search was performed for full-length NS3 protease, NS5A and NS5B polymerase sequences of HCV separated by genotypes 1a, 1b, 2a, 2b and 3a. These subtypes were chosen due to their worldwide prevalence and presence in drug trials, specifically genotype 1 with protease inhibitors (PI) and genotype 3 with polymerase inhibitor. Reference strains for the three genotypes were obtained (1a: AF009606, 1b: D90208, 2a: D00944, 2b: D10988 and 3a: D17763). Sequences containing missing data, such as gaps and sequencing errors, and sequences from patients previously treated with DAA were excluded from the analysis. To ensure the quality of the analysis, sequences with stop codons in the NS5B gene or with ambiguities consisting of more than 2 bases per nucleotide position or more than 2 ambiguities per codon at individual drug resistance-associated position were also excluded.

Alignment and edition of the sequences

The sequence alignment was performed with MEGA 6.06 MAC[13] followed by sequence editing, exclusion of sequences with missing data, and translation of the nucleic acids sequences into amino acids. The resulting protein sequences were then analyzed using BioEdit 7.2.5. to identify mutations associated with resistance[14].

Analysis of natural polymorphisms

Known mutations associated with resistance to protease-, NS5A complex- and polymerase-inhibitors were used to search for polymorphism patterns among HCV genotypes[15]. Only positions that have been described as being associated with failure in treatment in *in vivo* studies, and/or as conferring a more than 2-fold change in replication in comparison to the wildtype reference strain in *in vitro* phenotypic assays were included in the analysis.

RESULTS

Database search

The search resulted in 831 NS3, 869 NS5A and 6,065 NS5B sequences from HCV genotypes 1a, 1b, 2a, 2b and 3a. After the exclusion of incomplete sequences and those containing errors and/or gaps, and from patients previously treated with DAA, 798 sequences were included in the NS3 dataset. There were 313 from genotype 1a, 412 from genotype 1b, 19 from genotype 2a, 26 from genotype 2b and 28 from genotype 3a. There were 699 sequences identified in the NS5A dataset, with 272 from genotype 1a, 353 from genotype 1b, 19 from genotype 2a, 26 from genotype 2b and 29 from genotype 3a. For the NS5B polymerase there were 535 HCV sequences: 165 from genotypes 1a, 307 from genotype 1b, 19 from genotype 2a, 24 from genotype 2b and 20 from genotype 3a. Notably, the NS5B region has more than 5300 incomplete sequences deposited into this databank.

Mutation analyses

Mutation analyses were performed for positions

Table 1 Main amino acid substitutions found in the hepatitis C virus NS3 protease

	Amino acid (frequency %)											
	HCV genotype											
	1a			1b			2a		2b		3a	
Position	wt	Variants		wt	Variants		wt	Variants	wt	Variants	wt	Variants
36	V (98.08)	L (1.6)	M (0.32)	L (0.74)	I (0.25)	V (99.01)	L (100)	-	L (100)	-	L (100)	-
80	Q (54.37)	K (44.66)	R (0.97)	Q (93.37)	K (0.25)	L (6.39)	G (100)	-	G (100)	-	Q (100)	-
155	R (99.36)	K (0.64)	-	R (99.50)	P (0.50)	-	R (100)	-	R (100)	-	R (100)	-
156	A (100)	-	-	A (100)	-	-	A (100)	-	A (100)	-	A (100)	-
168	D (99.36)	E (0.32)	G (0.32)	D (98.77)	A (0.25)	E (0.98)	D (100)	-	D (100)	-	Q (100)	-

Amino acids in bold are associated with resistance. wt: Wild-type; HCV: Hepatitis C virus.

where resistance-associated amino acid substitutions have been described in the literature for conferring resistance to DAA. Amino acid substitutions related to HCV resistance to DAA are described below.

Frequency of resistance-associated variants

NS3/4A PI (Table 1): The available PI are more effective against HCV genotype 1 than to other genotypes due to natural polymorphisms in the NS3 region of the latter, therefore they are only used in the treatment of patients carrying HCV genotype 1. Thus, our analysis discusses mainly the findings for the genotype 1 dataset; nevertheless, the results for the other genotypes are shown in Table 1. The Q80K variant was the most prevalent mutation, found in 44.66% of the subtype 1a, and in 0.25% of subtype 1b sequences; the variant V80L was also observed in 6.39% of the latter. Other positions with frequencies higher than 2% were I170V (3.21%) in genotype 1a, and Y56F (15.93%), V132I (23.28%) and I170V (65.2%) in genotype 1b.

The V36L and V36M RAVs were identified in 1.6% and 0.32% of genotype 1a sequences, respectively, and in 0.74% and 0% of genotype 1b, respectively. The T54S variant was observed in 0.97% of genotype 1a and in 0.5% of genotype 1b sequences. The R155K variant was observed in 0.64% of genotype 1a sequences and was not observed in genotype 1b. There were two genotype 1b sequences (0.5%) with P substitution at position 155. Finally, no RAV A156T mutation was found in the 831 NS3 sequences analyzed.

The prevalence of resistant variants for the PI was found to be low in the dataset. Amino acid substitutions conferring resistance to these drugs were observed at NS3 position 168; the most frequent mutation was D168E, which was found in 0.32% of subtype 1a and in 0.98% of subtype 1b sequences. The prevalence of known NS3 variants enriched for by GZR was found to be low: F43S (0.31%) and Y56H (0%) in the whole dataset; the NS3 Q41R mutation was not observed.

NS5A replication complex inhibitors (Table 2): For subtype 1a there were a total of 272 NS5A sequences in our dataset. Mutations L23M (0.37%), M28T (0.75%), Q30H (1.47%), Q30R (0.37%), L31M (1.12%), P58S (2.21%) and Y93C (0.37%) were observed, whereas no variants were observed at NS5A position 32. Of the 353 subtype 1b sequences analyzed, 0.28% had the L23I mutation, 2.27% had L28M mutation, 5.95% had R30Q mutation, 3.40% had M31L mutation, 3.68% had P58S mutation, and 4.25% had the Y93H mutation. Of 19 subtype 2a sequences analyzed, one (5.26%) sequence had the Q30R mutation, 3 (15.79%) sequences had the M31L mutation, and one (5.26%) sequence had the H58P mutation. For subtype 2b a total of 26 sequences were analyzed, 6 (23.08%) with the L31M and one (3.85%) with the S58P mutation. In subtype 3a, for which 28 sequences were analyzed, the resistant variants M28I, A30L and P58R were found, each in a different sequence (3.57%) of the dataset. Only M31L was found in more than one sequence (23.08%) for this subtype. No mutation was found in the NS5A sequence at position 32 of any subtype.

NS5B polymerase inhibitors (Table 3): The NS5B S96T, C223H/Y, and S282T variants were not observed in any sequence in the present study, and the NS5B N142T variant was observed in 0.32% of the subtype 1b sequences. The V321L RAV was identified in 0.6% of genotype 1a sequences and in 0.32% of genotype 1b sequences.

The C316Y, S556G, and D559N RAVs were identified in 0.33%, 7.82% and 0.32% of genotype 1b sequences, respectively, and were not observed in other genotypes. The M414T and Y448H RAVs were not found in any of the 535 NS5B sequences analyzed.

Variants at NS5B positions 495 and 496 known to confer resistance polymerase inhibitors were not observed; on the other hand, the NS5B A421V and V499A substitutions were found in both subtypes 1a and 1b. The A421V mutation occurred in 9.64% of subtype 1a and in 4.55% of subtype 1b sequences. The V499A variant was the dominant amino acid substitution in subtype 1a sequences (95.15%), while for subtype 1b it was observed in only 9.74% of the sequences; there has been no reported evidence for negative clinical impact of the V499A.

Table 2 Main amino acid substitutions found in the hepatitis C virus NS5A protease

	Amino acid (frequency %)																							
	HCV genotype																							
	1a								1b								2a				2b				3a			
Position	wt								wt								wt				wt				wt			
28	M	94.38	I	0.37	T	0.75	V	4.49	L	97.17	M	2.27	V	0.52			F	100			L	100			M	96.46	I	3.57
30	Q	98.16	H	1.47	R	0.37			R	92.35	K	1.13	L/M	0.28	Q	5.95	K	94.74	R	5.26	K	100			A	96.46	L	3.57
31	L	98.88	M	1.12					L	96.03	M	3.40	I	0.57			M	84.21	L	15.79	M	76.92	L	23.08	L	100		
93	Y	98.90	C	0.37	H	0.74			Y	95.75	H	4.25					Y	100			Y	100			Y	100		

Amino acids in bold are associated with resistance. wt: Wild-type; HCV: Hepatitis C virus.

DISCUSSION

The resistance to direct antiviral therapy has been a major problem in a number of chronic viral infections. While much attention has been given to studies about HIV infection and resistance to antiviral therapy[16], the extent of mutations in the development of drug resistance in infection by HCV is less studied. The presence of HCV mutations is mainly due to factors such as selection pressure, error-prone replication (because of RNA polymerase's poor fidelity) and the high replication capacity of the virus. It is believed that any mutant can be generated continuously in a HCV-infected patient[17]. Hence, selected variants are considered to be pre-existent mutants generated during the natural HCV life cycle. The incidence of resistant variants is variable and depends on the binding domain, as well as on the different HCV populations, genotypes and subtypes.

With the exception of NS5B nucleoside analogues, the current DAAs target the NS3, as well as the allosteric sites of NS5B and NS5A, which all have a low threshold of resistance[10,18]. Data from both replicon analysis and from clinical trials have consistently identified viral mutations that can be associated with antiviral treatment failure[19]. A recently published analysis found that 58.7% of the HCV sequences deposited in GenBank harbored at least one dominant resistance variant[20]. In the present study, the overall prevalence of patients with variants resistant to DAAs was found to be low.

The frequency of variants resistant to NS5A-inhibitors ranged from 0% to 4.25%. Variants resistant to polymerase-inhibitors were observed mainly in genotype 1b and occurred in up to 7.82% (palm site), 2.41% (thumb site), and 9.74% (finger-loop site) of the sequences. These data corroborate other studies reported in the literature[15,21], and a comparasion with an analysis performed in Los Alamos databank[11] showed similar results (Table 4).

Pre-existing dominant resistance mutations in the NS3 region are more common in treatment-naïve patients infected with genotype 1a (cumulative incidence 8.6% *vs* 1.4%)[22]. Within NS3, the resistant Q80K mutation, which is based on available data only relevant for SMV and ASV, was the most prevalent (44.66% genotype 1a, 0.25% genotype 1b) and this result corroborates the recent findings of Pol *et al*[1,23] with European patients where, Q80K was observed in 34.7% and 2.1% of subtype 1a and 1b patients, respectively. The mutation I170V, present in 3.21% of genotype 1a and 65.20% of the genotypes 1b sequences analyzed, has been reported as not showing any influence on protease inhibitor activity[24]. Therefore, considering the actual recommendations in EASL and AASLD guidelines, up to 45% of patients with genotypes 1 have resistance mutations that can lead to treatment failure using PI.

The prevalence of resistant variants in the context of the NS5A inhibitors is highly dependent on viral subtype due to several positions having different baseline amino acids in each subtype[15]. Resistance against DVC, OMV, LDV is more common in genotype 1b (up to 4.25% of the sequences), but it can also occur in genotype 1a in less than 1.5% of the sequences. Furthermore, a broad cross-resistance between NS5A inhibitors is expected by the selection of mutations at codons 31 and/or 93 causing a loss in susceptibility to the majority of these compounds[24]. Other researchers also determined Y93H as most frequent baseline NS5A RAV in genotype 1b (6%-23%), followed by L31M (3%-4%)[24,25], whereas NS5A RAVs occurred at low frequencies in genotype 1a. Across the HCV genotypes, variation is observed at several of the residues identified as important sites for resistance, and substitutions M28L, Q30R, H58P that were found in genotype 1b; M28F, Q30K, L31M, H58P in genotype 2a; M28L, Q30K, L31M, H58P in genotype 2b and Q30A in genotype 3a could be defined as natural polymorphisms that distinguish those genotypes from 1a.

Table 3 Main amino acid substitutions found in the hepatitis C virus NS5A protease

	Amino acid (frequency %)														
	HCV genotype														
	1a				1b								2a	2b	3a
Position	wt				wt								wt	wt	wt
282	S	99.40	R	0.60	S								S	S	S
316	C				N	37.02	C	62.34	**R/Y**	**0.32**			C	C	C
556	S				N	89.24	D	0.98	**G**	**7.82**	N	1.95	G	G	G

Amino acid in bold are associated with resitance. wt: Wild-type; HCV: Hepatitis C virus.

Table 4 Resistance associated variants conferring resistance to direct antiviral agents[1]

	euHCVdb		Los Alamos	
	1a	1b	1a	1b
DAA	RAV	RAV	RAV	RAV
NS3				
Simeprevir	V36M (0.32%)		V36M (0.44%)	
	Q80K (44.66%)	Q80L (6.39%)	Q80K (36.62%)	Q80L (6.02%)
	S122G (4.49%)	S122G (9.07%)	n.a	n.a
	R155K (0.64%)		R155K (0.88%)	
	D168E (0.32%)	D168E (0.98%)	D168E (0.29%)	D168E (0.80%)
				D170T (0.20%)
Paritaprevir	R155K (0.64%)		R155K (0.88%)	
	D168E (0.32%)	D168E (0.98%)	D168E (0.29%)	D168E (0.80%)
Grazoprevir	A156T (0.00%)	A156T (0.00%)	A156T (0.00%)	A156T (0.00%)
	D168E (0.32%)	D168E (0.98%)	D168E (0.29%)	D168E (0.80%)
NS5A				
Ledipasvir	M28T (0.75%)		n.a	n.a
	Q30H (1.47%)			
	Q30R (0.37%)			
	L31M (1.12%)			
	Y93H (0.74%)	Y93H (4.25%)		
	Y93C (0.37%)			
Daclatasvir	M28T (0.75%)		n.a	n.a
	Q30H (1.47%)			
	Q30R (0.37%)			
	Y93H (0.74%)	Y93H (4.25%)		
Ombitasvir	M28V (4.49%)	Y93H (4.25%)	n.a	n.a
Elbasvir	Q30H (1.47%)			
	L31M (1.12%)			
	Y93H (0.74%)	Y93H (4.25%)		
NS5B				
Sofosbuvir	S282T (0.00%)	S282T (0.00%)	S282T (0.00%)	S282T (0.00%)
Dasabuvir		C316N (37.02%)		C316N (36.17%)
		C316Y (0.32%)		C316Y (0.30%)
		N556G (7.82%)	N556G (0.42%)	N556G (8.21%)

[1]DAAs recommended by the EASL and AASLD guidelines 2015. RAV: Resistance associated variants; DAAs: Direct antiviral agents; AASLD: American Association for the Study of Liver Diseases.

In contrast to NS3 PI, NS5B-non-nucleoside-inhibitors and NS5A-inhibitors where resistance mutations are subtype-dependent, little is known about NS5B nucleos(t)ide analogs genotype- and subtype-dependent resistance mutations. Several nucleotide analogs have shown very promising results and SOF is the first DAA in this drug class to gain regulatory approval[9,26], followed by DSV. In the present analysis, NS5B RAV were not detected in genotype 1a, whereas in genotype 1b, NS5B RAV were found in more than one third of the individuals (C316N in 37.02% and S556G in 7.82%) conferring low to medium resistance to DSV. In mixed cohorts consisting of American and European patients, while the S556G mutation was observed in frequencies of 0.5%-16%, the C316N RAVs occurred in frequencies lower than that observed in this study (11%-18%), at baseline in genotype 1b samples[15].

The S282T is the *in vitro* signature resistance mutation that conveys decreased susceptibility to SOF in the replicon system. Although the S282T substitution requires only a single nucleotide change, this variant was not found in any of the NS5B sequences analyzed in this study, neither in a previous study based on sequences from the Los Alamos databank[11]; in a study that analyzed 1459 HCV sequences from GenBank, this mutation was found in only one sequence[20].

With the currently in-use DAAs recommended by EASL and AASLD guidelines, our analyses suggest that it is possible that virologic failure could occur in half of the patients with HCV genotype 1a receiving SMV in combinations with pegylated-intereferon and ribavirin. In addition, more than 7% of the patients with HCV genotype 1b receiving DSV could also fail to respond to treatment, and the presence of variants with resistant mutations in the NS5A region should affect almost 5% of the treated individuals.

If in one hand the analysis of a public databank may not reflect the real prevalence of RAV in the population, on the other hand the abundance of information deposited in databank's sequences allows the identification of potentially unknown polymorphisms in populations not submitted to new HCV treatments. Since it is impossible to correlate criteria of inclusion in databanks with population data, epidemiological studies are necessary to determine the real prevalence of RVA in the population.

Although the potential to confer resistance to DAAs of the majority of the amino acid substitutions identified in our analyses is not known, the ability of HCV to rapidly evolve under drug selection pressure and the presence of baseline natural polymorphisms associated with resistance to DAA should be considered as possible threats to the success of these new therapies. The real impact of these constitutive RAV on the possibility of SVR with DAA remains unclear and undefined and some of these RAV apparently disappear after therapy (NS3/NS4 RAV) while others remain in the viral population (NS5 RAV). Globally, clinical significance of these constitutive RAV remains obscure

In summary, there are many relevant clinical questions that still need to be answered regarding HCV resistance to DAAs, mainly due to the limited available data and the large number of DAA approved or soon to be approved for clinical use. Perhaps resistance mutations in the new interferon-free DAA era may not have significant clinical impact initially[27,28], nonetheless the presence of a minor drug-resistance population will likely affect the success of the therapy upon the expansion and prolonged use of DAA regimens, and the relevance of pre-existing resistance mutations for responses to Interferon-free DAA therapies needs to be further investigated. Therefore, testing for drug resistance variants prior to the initiation of treatment will be needed in the very near future in order to help guide the selection of the most optimized treatment option.

COMMENTS

Background

Chronic hepatitis C virus (HCV) infection is a significant cause of morbidity and mortality worldwide. The main therapeutic targets against HCV are the viral NS3/4A protease, NS5B polymerase, and NS5A replication complex. While much attention has been given to HIV infection and resistance to antiviral therapy, the extent of mutations in the development of drug resistance in HCV infection is less studied. The presence of HCV mutations is mainly due to factors such as selection pressure, error-prone replication (because of RNA polymerase's poor fidelity) and the high replication capacity of the virus. It is believed that any mutant can be generated continuously in HCV-infected patients. Hence, selected variants are considered to be pre-existent mutations generated during the natural HCV life cycle. The incidence of resistant variants is variable and depends on the binding domain, as well as on the different HCV populations, genotypes and subtypes and pre-existence of resistance associated variants to direct antiviral agents (DAAs) reduces sustained virologic response rates. A recently published analysis found that 58.7% of the HCV sequences deposited in the GenBank harbored at least one dominant resistance variant.

Research frontiers

It is expected that in the near future a method able to detect all the mutations in the HCV genome will be available, making it possible to decide which DAA can be used to treat hepatitis C in a specific patient.

Innovations and breakthrough

This study showed a low frequency of mutations but a high number of polymorphisms of HVC genome which can impact in patients receiving treatment with DAAs.

Applications

Although the potential to confer resistance of the majority of the amino acid substitutions identified in our analyses is not known, the ability of HCV to rapidly evolve under drug selection pressure and the presence of baseline natural polymorphisms associated with resistance to DAAs should be considered as possible threats to the success of these new therapies.

Peer-review

This manuscript analyzed the occurrence of polymorphisms and resistant mutations in NS3, NS5A and NS5B regions in treatment-naive HCV sequences deposited in the European hepatitis C virus database.

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P- Reviewer: Bolhassani A, Cao GW, Grassi A, Lee HC, Rezaee-Zavareh MS
S- Editor: Gong ZM **L- Editor**: A **E- Editor**: Zhang FF

World Journal of Gastroenterology

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DOI: 10.3748/wjg.v22.i40.8918

World J Gastroenterol 2016 October 28; 22(40): 8918-8928
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

ORIGINAL ARTICLE

Basic Study

Antioxidant and anti-inflammatory action of melatonin in an experimental model of secondary biliary cirrhosis induced by bile duct ligation

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Author contributions: Colares JR and Marroni NP participated in the study design and development, analysis and interpretation of data and writing of the article; Schemitt EG, Hartmann RM, Licks F, Soares MC and Bosco AD participated in the oxidative stress and immunohistochemistry analyses and the interpretation of results.

Supported by Research and Event Promotion (FIPE) end accomplished in Hospital de Clínicas de Porto Alegre, No. 14-0474.

Institutional review board statement: The study was realized and approved by the Ethics Committee of HCPA (No. 14-0474).

Institutional animal care and use committee statement: All collections of biological samples and analyses carried out were in accordance with ethical principles of the Committee for Ethics on Animal Use (CEUA-HCPA [No. 14-0474]).

Conflict-of-interest statement: To the best of our knowledge, no conflict of interest exists.

Data sharing statement: No additional data are available.

Manuscript source: Invited manuscript

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Received: June 28, 2016
Peer-review started: June 29, 2016
First decision: August 8, 2016
Revised: August 24, 2016
Accepted: September 14, 2016
Article in press: September 14, 2016
Published online: October 28, 2016

Abstract

AIM

To evaluate the effects of melatonin (Mel) on oxidative stress in an experimental model of bile duct ligation (BDL).

METHODS

Male Wistar rats (n = 32, weight ± 300 g) were

allocated across four groups: CO (sham BDL), BDL (BDL surgery), CO + Mel (sham BDL and Mel administration) and BDL + Mel (BDL surgery and Mel administration). Mel was administered intraperitoneally for 2 wk, starting on postoperative day 15, at a dose of 20 mg/kg.

RESULTS

Mel was effective at the different standards, re-establishing normal liver enzyme levels, reducing the hepatosomatic and splenosomatic indices, restoring lipoperoxidation and antioxidant enzyme concentrations, reducing fibrosis and inflammation, and thereby reducing liver tissue injury in the treated animals.

CONCLUSION

The results of this study suggest a protective effect of Mel when administered to rats with secondary biliary cirrhosis induced by BDL.

Key words: Antioxidant; Cirrhosis; Fibrosis; Melatonin; Oxidative stress

Core tip: Secondary biliary cirrhosis is a late complication of prolonged extrahepatic bile duct obstruction that leads to structural and functional changes in the liver. Melatonin, the main product of the pineal gland, provides hepatic protection in the experimental model of bile duct ligation.

Colares JR, Schemitt EG, Hartmann RM, Licks F, Soares MC, Bosco AD, Marroni NP. Antioxidant and anti-inflammatory action of melatonin in an experimental model of secondary biliary cirrhosis induced by bile duct ligation. *World J Gastroenterol* 2016; 22(40): 8918-8928 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i40/8918.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i40.8918

INTRODUCTION

The liver has a complex structure, allowing it to play a key role in operation and maintenance of several vital functions of the organism, including synthesis activity and excretion of substances. In the liver lobes, hepatocytes are arranged in an orderly fashion out from a central vein, forming the sinusoids, from which they are separated by a narrow space (the space of Disse). This space is the site of the hepatic stellate cells (HSCs), which are known to possess contractile and fibrogenic properties, as well as the ability to synthesize extracellular matrix (ECM)[1-3].

Obstruction of the biliary tract is a congestive process that leads to numerous changes, such as ductular proliferation, stellate cell activation, and accumulation of ECM in the space of Disse. Occurrence of these changes may lead to the development of liver fibrosis, which, in turn, can lead to secondary biliary cirrhosis[4]. Cirrhosis of the liver represents the most advanced stage of fibrosis, in which there is evident loss of structure of the hepatic parenchyma. It is directly associated with development of septa and fibrotic nodules, changes in hepatic blood flow, and high risk of liver failure[5].

Studies have shown that HSCs are directly involved in the process of fibrosis formation and that their activation is influenced by products generated from lipid peroxidation (LPO), formation of reactive oxygen species (ROS), and presence of inflammatory mediators such as tumor necrosis factor-alpha (TNF-α), inducible nitric oxide synthase (iNOS), interleukins, and nuclear factor-kappa B[4,6].

As cirrhosis constitutes a major public health problem[7], much research is being conducted to develop and test different substances that could be used in its treatment. The objective of such substances aims to improve quality of life, increase survival, slow disease progression, and, possibly, mitigate the damage caused by formation of ROS and free radicals (FRs)[8,9].

Prolonged obstruction of the bile duct in rats is an experimental model for induction of secondary biliary cirrhosis[10]. In this model, the characteristic features of the disease are established at approximately 28 d[10]. Studies have demonstrated that the changes occurring in cirrhosis in human patients are similar to those found in experimental models, including jaundice, hepatomegaly, splenomegaly, abnormal gas exchange, and oxidative damage[11-15].

Melatonin (Mel; *N*-acetyl-5-methoxytryptamine) is the main product synthesized by the pineal gland, which produces Mel in a rhythmic manner, with production inhibited by light, so that its peak production occurs during the dark phase[16,17]. Several effects have been attributed to Mel, including antioxidant capacity, as well as anti-inflammatory and immunomodulatory properties[18-21].

There is an existing important link between cirrhosis, inflammation and oxidative stress; in this sense, treatments are required to protect the liver against these types of damage. Therefore, this present study investigated whether Mel (an anti-inflammatory agent and antioxidant) would afford hepatic-protection in an experimental model of cirrhosis.

MATERIALS AND METHODS

Animals

All animal procedures were conducted in accordance with the recommendations of the Health Research Ethics Committee of the Research and Graduate Studies Group at the Hospital de Clínicas de Porto Alegre (HCPA) in Brazil (approval number 14-0474), and as recommended in the Guide for the Care and Use of Laboratory Animals[22,23]. The sample comprised male Wistar rats n = 32, weight ± 300 g) that were

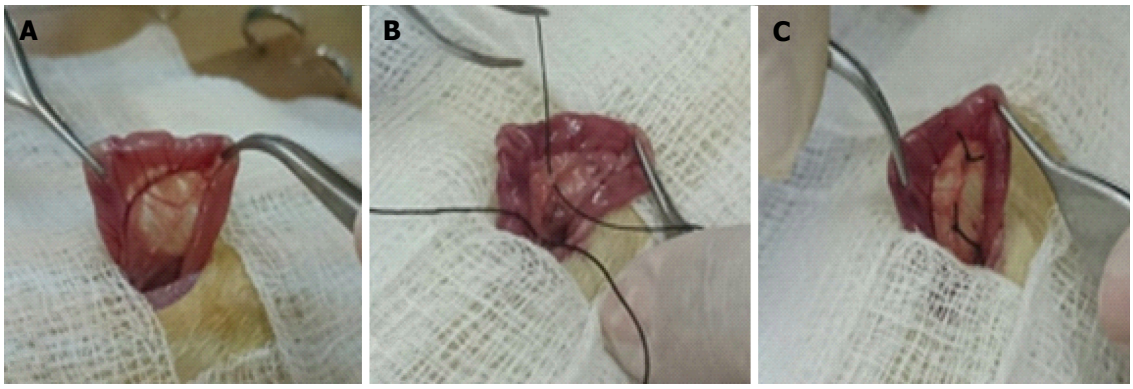

Figure 1 Bile duct ligation surgery. A: Localization of the bile duct; B: Passage of silk thread for duct isolation; C: Resection of the bile duct.

allocated across four groups: CO [sham bile duct ligation (BDL)], BDL (BDL surgery), CO + Mel (sham BDL and Mel administration) and BDL + Mel (BDL surgery and Mel administration). Cirrhosis was induced surgically by BDL as described by Kountouras *et al*[10].

Animal care and use statement

During the experiment, the animals were kept in boxes lined with wood shavings, under a 12-h light/dark cycle and controlled temperature conditions (18-22 ℃), with free access to water and chow. As shown in Figure 1A, animals in the CO and CO + Mel groups only underwent localization and manipulation of the bile duct (sham surgery). Figure 1B and C show the procedures performed in the BDL and BDL + Mel groups respectively: after localization of the bile duct, it was isolated and tied off with two knots made with 3-0 silk thread. All animals were euthanized at 29 d after the start of the experiment[24].

Administration of Mel

Treatment started on day 15 after BDL surgery. Mel was administered at a dose of 20 mg/kg body weight, always at 7:00 p.m., away from light.

Extraction of plasma

After the blood was collected through the retro-orbital plexus and placed in assay tubes with heparin, it was centrifuged at 4000 rpm for 10-min time. The precipitate was displaced and the plasma was removed with pipette (Labsystems 4500, 100-200 μL) for the different analyses of aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (AP) *via* commercial kit Labtest®.

Liver homogenates

For the preparation of the homogenate we used 9 mL of phosphate buffered solution (1.15% KCl) per gram of tissue (liver) and phenylmethylsulfonyl fluoride at a concentration of 100 mmol/L in isopropanol (10 μL/mL of KCl). The tissue was homogenized in ULTRA-TURRAX for 40 s at 0-2 ℃ and subsequently centrifuged for 10 min at 3000 rpm in a refrigerated centrifuge. The precipitate was discarded and the supernatant removed and frozen at -80 ℃ for subsequent biochemical analyses[25].

Liver enzyme activity

Activity of the liver enzymes AST and ALT, which are markers of hepatocyte integrity, were measured by the ultraviolet kinetic method. AP was measured by the colorimetric method. All tests were performed in plasma, under routine HCPA laboratory methods, using a Liquiform Labs® test commercial kit.

Hepatosomatic index and splenosomatic index

The liver and spleen were resected and weighed for derivation of the hepatosomatic index (HSI) and splenosomatic index (SSI), which were calculated as the percentage of total organ (liver and spleen) weight divided by the body weight of the animal: HSI = liver weight (g)/rat weight (g) × 100; SSI = spleen weight (g)/rat weight (g) × 100[26].

LPO

Liver tissue samples were placed in test tubes containing a mixture of trichloroacetic acid (TCA) 10% and thiobarbituric acid (TBA) 0.67%, heated at 100 ℃ in a water bath for 15 min, and cooled on ice for approximately 5 min. TBA reacts with LPO products to form a Schiff base, whereas TCA is used to denature proteins present and acidify the reaction. After cooling the samples, 1.5 mL of n-butyl alcohol was added to extract the formed pigment. Samples were stirred for 45 s and centrifuged for 10 min at 3000 rpm. Finally, the stained product present in the top fraction was read in a spectrophotometer at a wavelength of 535 nm. The TBARS concentration obtained was expressed as nmol/mg protein[27].

Activity of antioxidant enzymes and glutathione levels

Superoxide dismutase: The activity of superoxide dismutase (SOD) is defined by its ability to inhibit the reaction of superoxide radicals with adrenaline, and was monitored spectrophotometrically at 560 nm. Results were expressed as USOD/mg protein[28].

Catalase: The activity of Catalase (CAT) was determined by measuring the decrease in absorption in action medium containing 50 mmol/L phosphate buffered saline (pH 7.2) and 0.3 mol/L hydrogen peroxide. The enzyme activity was assayed spectrophotometrically at 240 nm and expressed as pmol/mg protein[29].

Glutathione peroxidase: The activity of the antioxidant enzyme glutathione peroxidase (GPx) was assessed by the NADPH oxidation rate in the presence of reduced glutathione (GSH) and glutathione reductase. Sodium azide was added to inhibit CAT activity. The enzyme activity was measured spectrophotometrically at 340 nm and expressed as nmol/min/mg protein[30].

Glutathione S-transferase: The glutathione S-transferase (GST) activity assay is based on an enzyme reaction which at 30 ℃ catalyzes the formation of 1 μmol DNP-SG using a GSH concentration of 1 mmol/L and chloro dinitrobenzene (CDNB). The enzyme activity was measured spectrophotometrically at 340 nm and expressed as μmol/min/mg protein[31].

GSH reduced: To prepare the homogenate for measuring levels of GSH reduced, for every 1 g of tissue, 20 mL of perchloric acid (2 mmol/L) + EDTA (4 mmol/L) was diluted in 1 mL H_2O. The levels GSH were evaluated spectrophotometrically at 412 nm by quantifying intracellular levels of GSH from modification of 2-nitrobenzoic acid and expressed as μmol/mg protein[32].

Histological analysis

After anatomical dissection of the liver of each animal, approximately 2 cm were removed for histological evaluation. The tissues were isolated and immersed in 10% buffered formalin for 24 h for fixation, followed by histological processing (dehydration in a graded alcohol series of six concentrations, clearing in xylol at two concentrations, and embedding in paraffin at 64 ℃). The resulting paraffin blocks were attached to a microtome (Leitz® 1512) and slices of 3 μm thickness were obtained. These specimens were placed in a histological bath at 50 ℃. For the staining step, the slides were immersed in vats containing hematoxylin-eosin (HE) and Picrosirius red (5 min in each stain). After the hydration stage, the sample was covered with a coverslip and fixed with Canada Balsam or the blade, finalizing the preparation process. The slides were examined by a pathologist who was blinded to group allocation and were photographed under a NIKON LABOPHOT binocular microscope at 200 × magnification.

Immunohistochemistry (iNOS and TNF-α)

For immunohistochemistry, liver tissue samples were fixed in 10% formalin and placed in a histological tissue processor (ANCAP), through a graded ethanol series and two vats of xylene, for dehydration. Specimens were then embedded and blocks were cooled, modeled, and attached to a microtome (Leitz® 1512) to obtain slices 4 μm thick. The resulting slides were incubated with mouse anti-iNOS (SC-7271; Santa Cruz Biotechnology, Santa Cruz, CA, United States) and TNF-α polyclonal antibodies (SC-52746; Santa Cruz Biotechnology) at a dilution of 1:200 overnight at 4° C, followed by incubation with the secondary antibody (SC-2005; Santa Cruz Biotechnology) at 1:300 for 30 min at room temperature. The slides were analyzed by a pathologist who was blinded to group allocation and were photographed under a NIKON LABOPHOT binocular microscope at 200 × magnification. Digital images were analyzed in Image-Pro Plus version 4.5 (Media Cybernetics, Rockville, MD, United States). The expression level was determined by multiplying the average density of the image by the percent area positively stained by the antibodies [brown colored areas obtained by the peroxidase + diaminobenzidine reaction].

Ethical consideration

The present study was accomplished in the HCPA with the approval of the project (No. 14-0474).

Animal care and use statement

All experimental design, collections of biological samples and analyses carried out were in accordance with ethical principles of the Committee Ethics on Animal Use (CEUA-HCPA).

Statistical analysis

Quantitative data are presented as mean ± SD error. The comparison between groups was performed by one-way analysis of variance followed by the Student-Newman-Keuls procedure. $P < 0.05$ was considered as statistically significant.

RESULTS

Liver enzyme activity

Evaluation of liver enzyme activity performed in plasma showed a significant increase in all enzymes in the BDL group compared with the control groups, as well as a significant reduction of these values in the BDL + Mel group compared to the BDL group. AST levels increased 379% in the BDL group compared to the CO group, and were 72% reduced in the BDL + Mel group compared to the BDL group. ALT, a specific marker of liver damage, was 186% increased in the BDL group in relation to the CO group and 60% lower in the BDL + Mel group compared to the BDL group. AP levels were 211% higher in the BDL group compared to the CO group and 72% lower in the BDL + Mel group compared to the BDL group ($P < 0.001$) (Table 1).

Table 1 Plasma levels of aspartate aminotransferase, alanine aminotransferase, and alkaline phosphatase in the different experimental groups

Group	AST (U/L)	ALT (U/L)	AP (U/L)
CO	88.8 ± 0.07	37.0 ± 1.9	122.4 ± 13.5
CO + Mel	90.4 ± 8.4	38.8 ± 3.2	111.6 ± 8.1
BDL	425.8 ± 46.6[e]	105.8 ± 13.5[e]	381.2 ± 35.5[e]
BDL + Mel	117.5 ± 18.8[f]	42.0 ± 3.4[f]	104.3 ± 11.03[f]

All concentrations are expressed as mean ± SD error. Significant difference exists between the BDL and control groups (CO and CO + Mel) ($^eP < 0.001$). Significant difference exists between the BDL and BDL + Mel groups ($^fP < 0.001$). CO: Control; CO + Mel: Control + melatonin; BDL: Bile duct ligation; BDL + Mel: Bile duct ligation + melatonin.

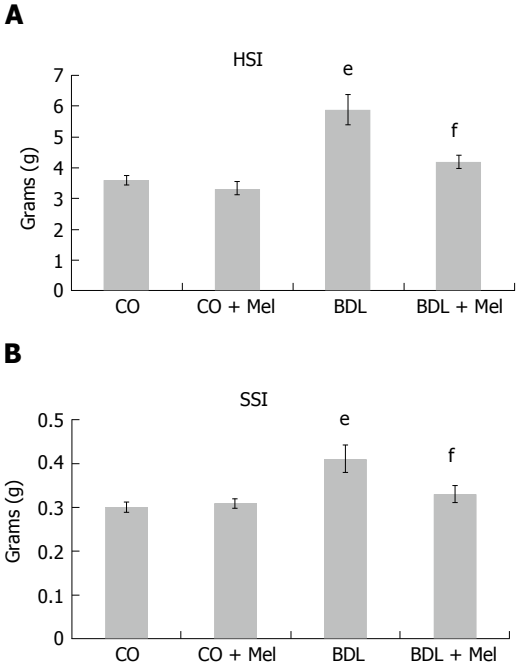

Figure 2 Mean hepatosomatic and splenosomatic index values in the different experimental groups. All results are expressed as mean ± SD error. Significant difference existed between the BDL and control groups (CO and CO + Mel) ($^eP < 0.001$). Significant difference existed between the BDL and BDL + Mel groups ($^fP < 0.001$). CO: Control; CO + Mel: Control + melatonin; BDL: Bile duct ligation; BDL + Mel: Bile duct ligation + melatonin; HSI: Hepatosomatic index; SSI: Splenosomatic index.

HSI and SSI

Analysis of HSI and SSI showed significant increases in the BDL group compared to control animals (CO and CO + Mel), as well as a significant decrease in the BDL + Mel group compared to the cirrhotic group (BDL) (Figure 2A and B).

Lipoperoxidation and GSH levels

The evaluation of LPO and GSH levels was performed on homogenized liver.

The LPO analysis revealed a significant increase in LPO markers in the BDL group compared to the CO and CO + Mel groups, and administration of Mel to BDL + Mel animals was associated with a significant decrease in damage in this group. GSH levels were

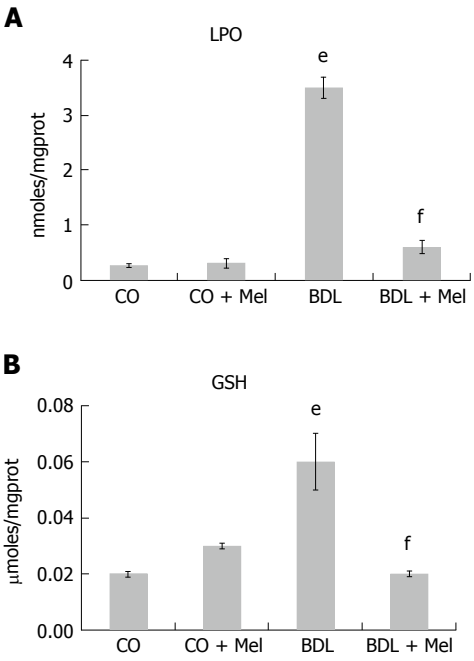

Figure 3 Lipoperoxidation markers and glutathione levels in the different experimental groups. All results are expressed as mean ± SD error. Significant difference exists between the BDL and control groups (CO and CO + Mel) ($^eP < 0.001$). Significant difference exists between the BDL and BDL + Mel groups ($^fP < 0.001$). CO: Control; CO + Mel: Control + melatonin; BDL: Bile duct ligation; BDL + Mel: Bile duct ligation + melatonin; GSH: Glutathione; LPO: Lipid peroxidation.

increased in the BDL group compared to the control groups (CO and CO + Mel), and reduced in BDL + Mel compared to BDL (Figure 3A and B).

Antioxidant enzyme activity

Evaluation of SOD, CAT, GPx and GST activity revealed reductions in SOD and CAT in BDL animals compared to controls (CO and CO + Mel), as well as functional recovery of these enzymes in the BDL + Mel group compared to the BDL group. Activity of GPx and GST were increased in the BDL group compared to both control groups (CO and CO + Mel), and decreased in the BDL + Mel group compared with BDL (Table 2).

Histological analysis

HE staining: In the control groups (CO and CO + Mel), histological analysis by HE staining revealed normal liver parenchyma with clearly defined hepatocyte cords. In the BDL group, there was tissue disorganization with loss of hepatocyte cords and inflammatory infiltration. In the cirrhotic group treated with Mel (BDL + Mel), restructuring of these patterns was observed, with formation of hepatocyte cords arising from a centrilobular vein (Figure 4).

Picrosirius staining: Assessment of liver fibrosis in Picrosirius-stained sections revealed absence of fibrotic septa in the control groups (CO and CO + Mel). In animals subjected to BDL, there was positive labeling consistent with presence of fibrotic septa. However, in

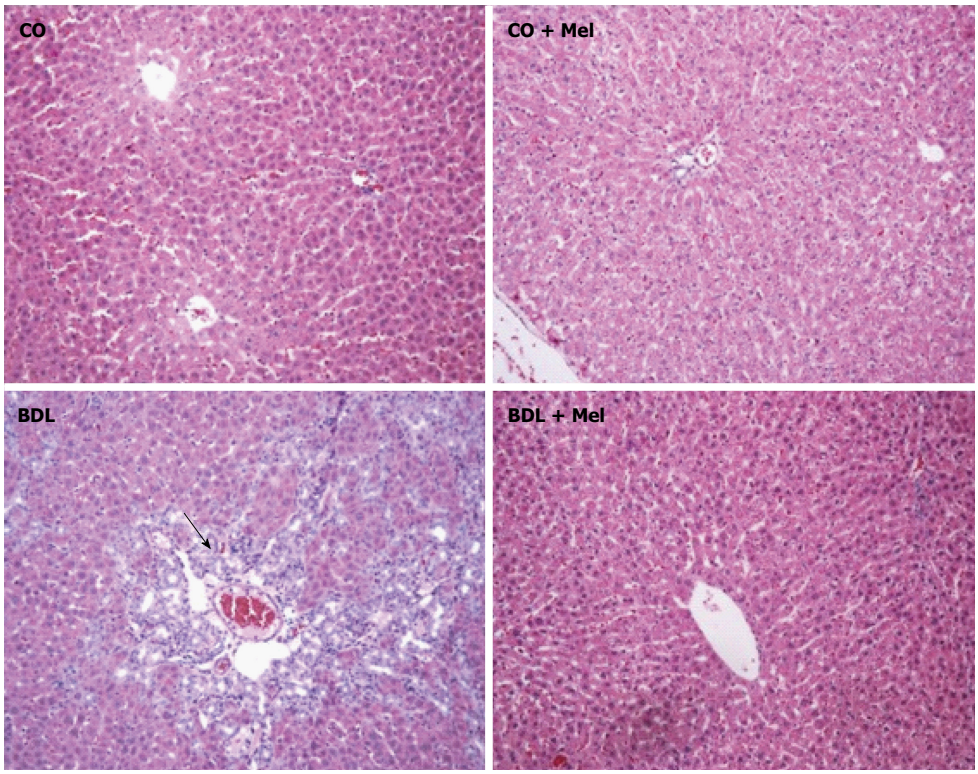

Figure 4 Histological analysis of liver tissue in the different experimental groups. HE staining, 200 × magnification. The arrow indicates the presence of inflammatory infiltrate. CO: Control; CO + Mel: Control + melatonin; BDL: Bile duct ligation; BDL + Mel: Bile duct ligation + melatonin.

Table 2 Activity of antioxidant enzymes in the different experimental groups

Groups	SOD (USOD/mg prot)	CAT (pmol/mg prot)	GPx (nmol/min/mg prot)	GST (μmol/min/mg prot)
CO	2.43 ± 0.17	2.19 ± 0.21	6.93 ± 0.76	2.28 ± 0.20
CO + Mel	2.31 ± 0.25	2.21 ± 0.28	7.15 ± 1.05	2.57 ± 0.09
BDL	0.88 ± 0.21[1]	1.09 ± 0.01[1]	37.78 ± 2.39[1]	5.08 ± 0.43[1]
BDL + Mel	2.47 ± 0.22[2]	2.46 ± 0.04[2]	9.61 ± 1.20[2]	1.93 ± 0.21[2]

All values are expressed as mean ± SD error. [1]Significant difference exists between the BDL and control groups (CO and CO + Mel) (SOD, $P < 0.01$; CAT, $P < 0.05$; GPx and GST, $P < 0.001$); [2]Significant difference exists between the BDL and BDL + Mel groups (SOD and CAT, $P < 0.01$; GPx and GST, $P < 0.001$). CO: Control; CO + Mel: Control + melatonin; BDL: Bile duct ligation; BDL + Mel: Bile duct ligation + melatonin. SOD: Superoxide dismutase; CAT: Catalase; GPx: Glutathione peroxidase; GST: Glutathione S-transferase.

the BDL + Mel group, fibrosis was minimal (Figure 5).

Immunohistochemistry and quantification of iNOS and TNF-α

Liver specimens from the BDL group exhibited strong positive staining for iNOS (Figure 6) and TNF-α (Figure 7), whereas specimens from the CO and CO + Mel groups did not stain. Treatment with Mel reduced iNOS and TNF-α positivity. Likewise, iNOS and TNF-α expression was significantly reduced in BDL + Mel compared to the BDL group ($P < 0.001$; Figures 6 and 7).

DISCUSSION

The BDL model is widely used to reproduce secondary biliary cirrhosis in animals, as it induces changes that closely resemble those seen in cirrhosis in humans and in experimental cirrhosis induced by carbon tetrachloride (CCl_4)[4,10,12].

Liver integrity can be evaluated by measuring levels of the enzymes AST, ALT, and AP. Increases in these markers suggest liver dysfunction[32]. In the present study, animals subjected to BDL exhibited higher levels of AST, ALT, and AP than animals in all other groups. Our findings also demonstrated that administration of Mel to animals with cirrhosis induced by BDL reduced the liver damage caused by duct ligation. These results corroborate the findings of a previous study conducted by Bona *et al*[4], using the CCl_4 model of cirrhosis, in which animals exhibited a significant increase in AST, ALT and AP levels and

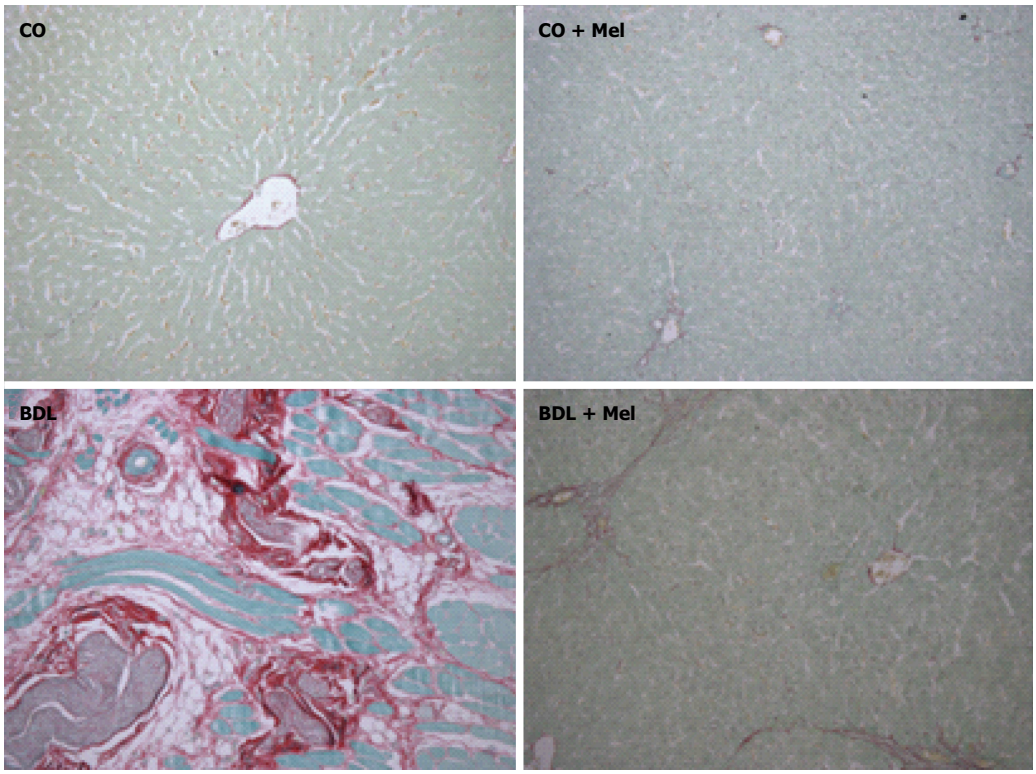

Figure 5 Histological analysis of liver tissue in the different experimental groups. Picrosirius staining, 200 × magnification. CO: Control; CO + Mel: Control + melatonin; BDL: Bile duct ligation; BDL + Mel: Bile duct ligation + melatonin.

equally significant reductions of these markers after treatment with the antioxidant quercetin. Shu *et al*[9] demonstrated that administration of tanshinone IIA, the active ingredient of *Salvia miltiorrhiza*, reduced ALT and AST levels in an experimental model of cirrhosis in rats.

The terms hepatomegaly and splenomegaly refer, respectively, to enlargement of the liver and spleen. Hepatomegaly is often associated with hepatobiliary diseases. Splenomegaly, in turn, is associated with numerous chronic diseases of the liver[33,34,35]. In our study, both the HSI and SSI were significantly increased in the BDL group compared with both control groups, and both indices decreased to near control levels when administered Mel in the BDL + Mel group.

The splenomegaly observed in the BDL model is due to portal hypertension as a result of enlargement of the splenic veins. Hepatomegaly, in turn, is secondary to biliary retention and subsequent obstruction of biliary drainage, which ultimately leads to liver fibrosis[13,14,36]. Using a model of liver damage induced by administration of polychlorinated biphenyls, Oliveira *et al*[33] found that splenomegaly was minimal in exposed animals given the antioxidant quercetin. LPO causes disorganization of cell membranes, resulting in an increase in membrane permeability and consequent extravasation of enzymes, leading to cell death[37]. Studies have demonstrated that MDA levels may be associated with increased LPO[5].

Studies report that, in the pathophysiology of biliary cirrhosis, liver damage is maximized by the action of FRs[12]. This phenomenon was also observed in the present study by measuring LPO, which was significantly higher in the cirrhotic group (BDL) when compared to the other groups and, accordingly, may have been associated with a process of cell membrane damage. Furthermore, the BDL + Mel group exhibited a significant decrease in LPO as compared with the BDL group, which suggests a protective role of Mel against LPO induced by BDL. These data corroborate a previous study by Bona *et al*[4] (2012), in which LPO was found to be increased in a model of CCl_4-induced cirrhosis, and quercetin treatment appeared to decrease LPO significantly.

CAT catalyzes the breakdown of H_2O_2 into water and O_2. SOD is regarded as the first line of defense against ROS formation, and decreases in its activity could be related to increased LPO and heightened consumption of the enzyme in an attempt to decrease oxidative damage from ROS dismutation and H_2O_2 formation[38].

In the present study, activity of the antioxidant enzymes SOD and CAT was significantly decreased in the BDL group compared to all others, and Mel administration was able to restore activity of these enzymes to near-control levels. These data suggest that treatment with Mel attenuated FR formation secondary to liver damage resulting from BDL-induced cirrhosis. These data corroborate the findings of Bona *et al*[4] (2012), a study in which rats with CCl_4-induced

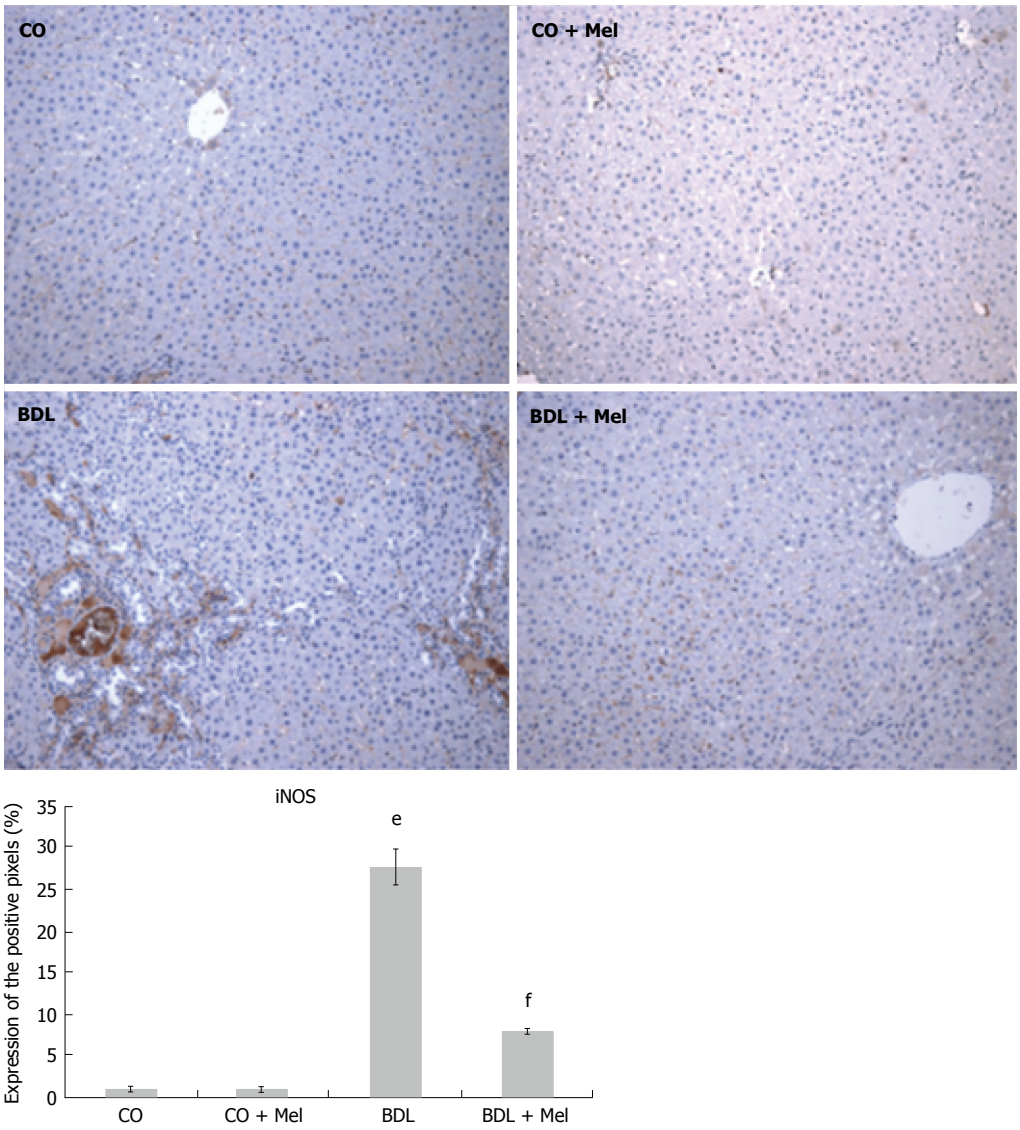

Figure 6 Expression of inducible nitric oxide synthase in the different experimental groups. Magnification 200 ×. All values are expressed as mean ± SD error. Significant difference exists between the BDL and control groups (CO and CO + Mel) ($^eP < 0.001$). Significant difference exists between the BDL and BDL + Mel groups ($^fP < 0.001$). CO: Control; CO + Mel: Control + melatonin; BDL: Bile duct ligation; BDL + Mel: Bile duct ligation + melatonin; iNOS: Inducible nitric oxide synthase.

cirrhosis exhibited an increase in antioxidant enzymes after treatment with quercetin.

Levels of the other enzymes evaluated (GPx and GST), as well as of GSH, were increased in the BDL group compared to the other groups, and decreased significantly to near-control values in the BDL + Mel group. The increases observed in cirrhotic animals may be associated with enzyme activation in an attempt to clear FRs and minimize oxidative damage from the disease, while the reduction in these levels in the group administered Mel suggests decreased FR formation[39]. These data corroborate the findings of Amália *et al*[38], who observed that, in a model of CCl_4-induced cirrhosis, GPx, GST and GSH levels were increased, and treatment with quercetin appeared to decrease these values.

Changes in the hepatic parenchyma, as well as formation of fibrotic septa and necrosis, are often associated with the cirrhotic process[4]. In our study, we observed loss of tissue organization in the BDL group when assessed by HE staining, demonstrating cellular disorganization with loss of hepatocyte cords and presence of inflammatory infiltrate. In the BDL + Mel group, a restructuring effect was observed, with tissue organization resembling that seen in the CO and CO + Mel groups.

Ferrari *et al*[40] demonstrated that rats with cirrhosis, whether induced by BDL or by CCl_4, exhibit necrosis, fibrotic nodules, inflammatory infiltrate and cellular changes. Tieppo *et al*[14] also observed that rats subjected to BDL exhibit hepatic changes with ductular proliferation and fibrosis, findings that improved in cirrhotic rats treated with quercetin.

Fibrosis is the end result of long-term liver injury. Evaluation of fibrotic area in Picrosirius-stained slides revealed increased collagen deposition in the BDL group,

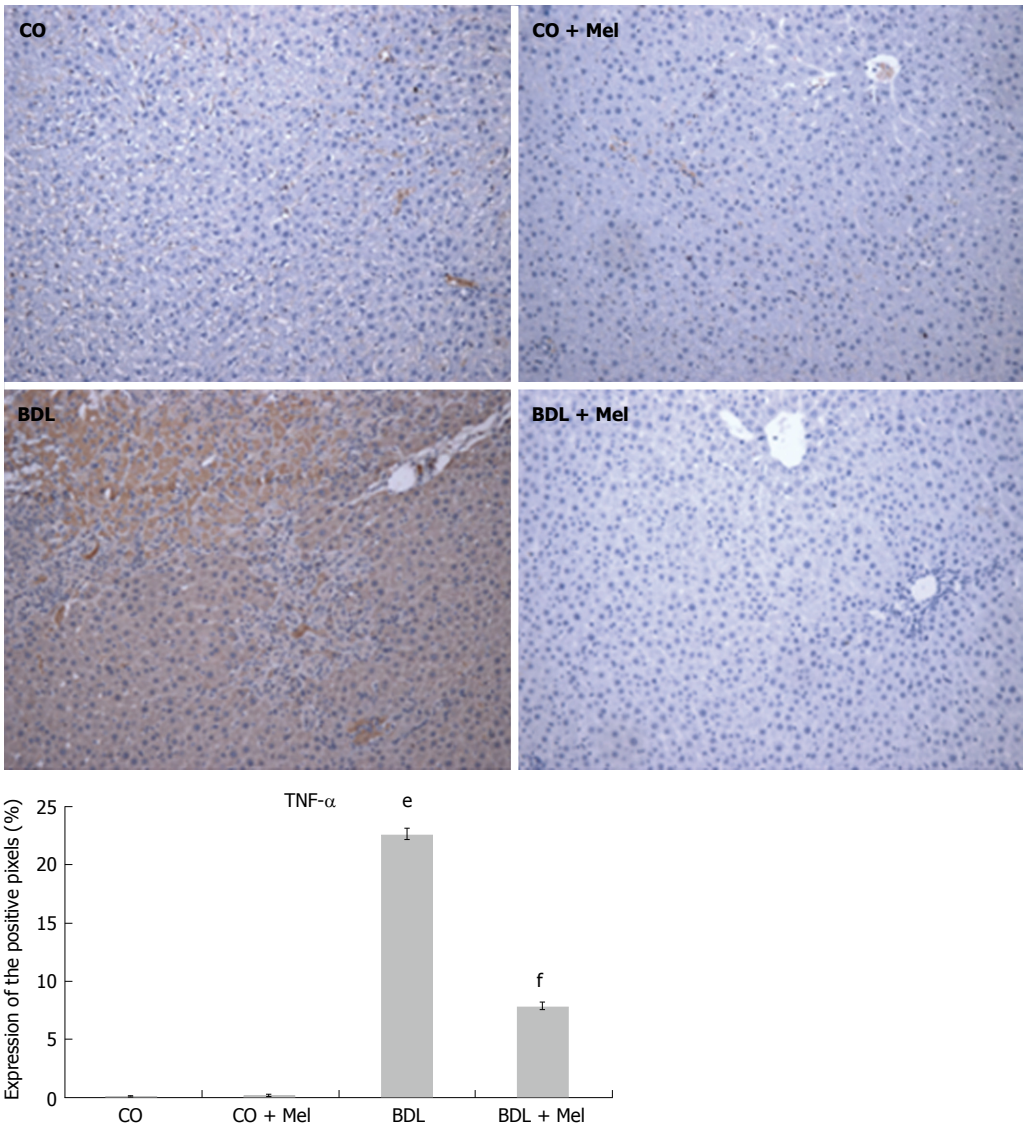

Figure 7 Expression of tumor necrosis factor in the different experimental groups. Magnification 200 ×. All values are expressed as mean ± SD error. Significant difference exists between the BDL and control groups (CO and CO + Mel) ($^eP < 0.001$). Significant difference exists between the BDL and BDL + Mel groups ($^fP < 0.001$). CO: Control; CO + Mel: Control + melatonin; BDL: Bile duct ligation; BDL + Mel: Bile duct ligation + melatonin; TNF-α: Tumor necrosis factor-alpha.

in contrast to the BDL + Mel animals, in which collagen deposition was minimal. These data corroborate various studies which observed increased collagen deposition in the liver of rats with cirrhosis induced by CCl_4 and BDL[4,14]. Saleh *et al*[41] administered the natural marine compound *Sepia officinalis*, known for its major antioxidant, antibacterial and antitumor effects, and observed a reduction in collagen deposition in animals subjected to bile duct ligation.

Increased production of TNF-α and iNOS is related to acute and chronic inflammatory processes. In the present study, we found higher TNF-α and iNOS expression in animals subjected to BDL, as well as decreased expression of these parameters in animals administered Mel. These findings corroborate those of Gonçalves Schemitt *et al*[42], who observed lower expression of TNF-α and iNOS in animals treated with glutamine in an experimental model of fulminant hepatic failure.

In view of the evidence presented herein, we suggest that the antioxidant and anti-inflammatory effects of Mel acted to restore serum levels of liver enzymes and the HSI and SSI, decrease LPO, restore antioxidant enzymes, and attenuate collagen deposition, inflammation and tissue damage in the livers of animals subjected to BDL. However, other pathways of Mel action should be studied to elucidate the protective mechanisms involved in this experimental model.

ACKNOWLEDGMENTS

The authors would like to thank the Coordination for the Higher-Level Personnel (CAPES), the Research Support Foundation of Rio Grande do Sul (FAPERGS), the National Council of Research Development (CNPq),

the Federal University of Rio Grande do Sul (UFRGS) and the Lutheran University of Brazil (ULBRA) for their support.

COMMENTS

Background

Liver cirrhosis is characterized by the appearance of septa and fibrotic nodules. Bile duct ligation (BDL) in rats is an effective experimental model of secondary biliary cirrhosis induction. Melatonin (Mel) has proven to be a potent antioxidant in different experimental models.

Research frontiers

Experiments previous studies have proved that Mel presents itself as a potent antioxidant in different experimental models.

Innovations and breakthroughs

This is the first study evaluating the antioxidant capacity of Mel in a surgical model of secondary biliary cirrhosis, in order to evaluate its possible therapeutic efficacy.

Applications

Despite the secondary biliary cirrhosis affect, a significant number of patients, still do not have an effective treatment. These data indicate that Mel administration may be a target for further study and suggest its applicability for patients in order to better support the life of the same.

Terminology

N-acetyl-5-methoxytryptamine, a physiologic hormone synthesized in a rhythmic manner by the pineal gland and with production inhibited by light. Exogenous administration has been related to its antioxidant capacity, and anti-inflammatory and immunomodulatory properties.

Peer-review

This manuscript is a good research article. The study is interesting and appropriate because it provides novel information about the beneficial effects of Mel on a model of cirrhosis. The authors studied the antioxidant and anti-inflammatory effects of a treatment with the indoleamine and evaluated the possible reversion of the structural changes induced in the liver by BDL.

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P- Reviewer: Chen WX, Maarman G, Vega-Naredo I **S- Editor**: Qi Y
L- Editor: Filipodia **E- Editor**: Zhang FF

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DOI: 10.3748/wjg.v22.i40.8929

World J Gastroenterol 2016 October 28; 22(40): 8929-8939
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

ORIGINAL ARTICLE

Basic Study

Fecal microbiota in pouchitis and ulcerative colitis

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Author contributions: Li KY conceived and designed the study, acquired the data and contributed to final approval of the version of the article to be published; Wang JL performed the majority of the experiments and analyzed the data; Wei JP and Wang LT analyzed and interpreted the data; Wang JL drafted the manuscript; Gao SY and Zhang YY performed the statistical analysis and drafted the manuscript; Liu G revised the manuscript for important intellectual content, and supplied administrative, technical, and material support.

Supported by Academician Jie-Shou Li Intestinal Barrier Research Foundation, No. LJS_201008.

Institutional review board statement: The study was reviewed and approved by Tianjin Medical University General Hospital Institutional Review Board, Tianjin 300052, China.

Conflict-of-interest statement: This work was presented as a poster at the 10th International Congress on Autoimmunity in Leipzig, Germany, April 6-10, 2016. We have no financial relationships to disclose.

Data sharing statement: No additional data are available.

Manuscript source: Invited manuscript

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Received: June 24, 2016
Peer-review started: June 24, 2016
First decision: August 8, 2016
Revised: August 15, 2016
Accepted: August 30, 2016
Article in press: August 30, 2016
Published online: October 28, 2016

Abstract

AIM

To investigate the changes in microbiota in feces of patients with ulcerative colitis (UC) and pouchitis using genomic technology.

METHODS

Fecal samples were obtained from UC patients with or without an ileal pouch-anal anastomosis (IPAA) procedure, as well as healthy controls. The touchdown polymerase chain reaction technique was used to amplify the whole V3 region of the 16S rRNA gene, which was transcribed from DNA extracted from fecal samples. Denaturing gradient gel electrophoresis was used to separate the amplicons. The band profiles and similarity indices were analyzed digitally. The predominant microbiota in different groups was confirmed by sequencing the 16S rRNA gene.

RESULTS

Microbial biodiversity in the healthy controls was significantly higher compared with the UC groups (P < 0.001) and IPAA groups (P < 0.001). Compared with healthy controls, the UC patients in remission and those in the mildly active stage, the predominant species in patients with moderately and severely active UC changed obviously. In addition, the proportion of the dominant microbiota, which was negatively correlated with the disease activity of UC (r = -6.591, P < 0.01),

was decreased in pouchitis patients. The numbers of two types of bacteria, *Faecalibacterium prausnitzii* and *Eubacterium rectale*, were reduced in UC. Patients with pouchitis had an altered microbiota composition compared with UC patients. The microbiota from pouchitis patients was less diverse than that from severely active UC patients. Sequencing results showed that similar microbiota, such as *Clostridium perfringens*, were shared in both UC and pouchitis.

CONCLUSION

Less diverse fecal microbiota was present in patients with UC and pouchitis. Increased *C. perfringens* in feces suggest its role in the exacerbation of UC and pouchitis.

Key words: Pouchitis; Intestinal flora; Ulcerative colitis; Disease activity index; Ileal pouch-anal anastomosis

Core tip: Dysbiosis in pouchitis might be similar to that observed in ulcerative colitis (UC). This study aimed to determine the altered microflora in patients with UC and pouchitis, and to investigate the relationship between them. We demonstrated the reduced biodiversity of the fecal microbiota in UC and pouchitis patients. The altered composition of the intestinal microbiota in UC and pouchitis included decreased numbers of two bacteria commonly observed in UC, and higher levels of *Clostridium perfringens* in both UC and pouchitis. The increase of this bacterium in feces suggested that it plays a role in exacerbating UC and pouchitis.

Li KY, Wang JL, Wei JP, Gao SY, Zhang YY, Wang LT, Liu G. Fecal microbiota in pouchitis and ulcerative colitis. *World J Gastroenterol* 2016; 22(40): 8929-8939 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i40/8929.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i40.8929

INTRODUCTION

One of the most common complications in ulcerative colitis (UC) patients who undergo ileal pouch-anal anastomosis (IPAA) surgery is pouchitis[1]. Interestingly, it is rarely seen in postoperative patients suffering from familial adenomatous polyposis. The gut microbiome plays a vital role in UC[2]. Antibiotics and probiotics are used to treat and prevent pouchitis[3]. The gut microbiome might play a vital role in the pathogenesis of UC[4].

However, direct evidence of the role of microflora in pathogenesis of pouchitis is lacking. Studies have shown variation in the microbiota in pouchitis and healthy controls; however, based on different culture methods and molecular biology techniques, no consensus was available[3]. Johnson *et al*[5] and Lim *et al*[6] showed no differences between pouchitis and no pouchitis (NP) groups. Some studies have suggested a reduction in bacterial diversity in pouchitis but not dysbiosis[7]. Other studies revealed an increase in bacterial diversity in pouchitis[8], such as increased numbers of *Clostridium* and *Eubacterium*[9], while others showed less *Enterococcaceae* in pouchitis[10]. The findings of the most recent study revealed that disorders caused by protective and harmful bacteria are associated with pouch inflammation[11]. The emergence of *Ruminococcus gnavus* (R. *gnavus*), *Bacteriodes vulgatus* and *Clostridium perfringens* (*C. perfringens*) and deficiency of *Blautia* and *Roseburia* in patients with UC before IPAA is closely related to pouchitis[12].

Denaturing gradient gel electrophoresis (DGGE) was reported to be useful to analyze changes in the composition of the intestinal microbiota[2]. We hypothesized that dysbiosis occurring in the pouch might be similar to that observed in UC. Thus, we determined the altered microflora in pouchitis and UC patients, and investigated the relationship between them.

MATERIALS AND METHODS

Patients and fecal samples

Patients who underwent IPAA for UC were recruited. Pouchitis was diagnosed based on symptoms, endoscopy and histology of the pouch. Patients underwent pouch endoscopy and biopsy. Physicians recorded clinical data, the pouch appearance and pathological manifestations based on the pouchitis disease activity index (PDAI)[13]. Antibiotic or other drug therapy was stopped to prevent variations in the microbiome 4 wk before collecting the fecal sample. A limited number of patients with pouches were excluded from the study because of antibiotic or probiotic usage for pouchitis or severe concomitant disease.

According to the PDAI, patients with IPAA were divided into two groups: NP, PDAI < 7 points (n = 11) and pouchitis, PDAI ⩾ 7 points (n = 8). Matched fecal samples were obtained from healthy controls (n = 16) and from 41 UC patients who did not undergo IPAA. All the UC patients without a pouch underwent endoscopy. The Mayo scoring system for assessment of ulcerative colitis activity was employed to divide patients with UC into the remission group (n = 10), mild activity group (n = 11), moderate activity group (n = 10) and severe activity group (n = 10).

All fecal samples were collected at the hospital and preserved at 4 ℃. Upon arrival at the laboratory, the samples were frozen at -80 ℃ within 12 h. This study was reviewed and approved by the Tianjin Medical University General Hospital Ethical Committee (China). Patient data are summarized in Table 1.

Fecal DNA extraction

A Fecal DNA kit (Aidlab Biotechnologies Co, Ltd,

Table 1 Demographic and clinical characteristics of the subjects

	Healthy controls	UC (n = 41)				Pouch (n = 19)	
		Remission	Mild	Moderate	Severe	Pouchitis	NP
Number of patients	16	10	11	10	10	8	11
Sex, n, M/F	9/7	5/5	7/4	5/5	6/4	5/3	5/6
Age (yr)	46.2 ± 10.5	41.8 ± 9.2	43.9 ± 10.5	46 ± 8.2	40.8 ± 11.3	44.9 ± 15.6	47.8 ± 13.2
UC duration (yr)	NA	5.0 ± 1.6	7.0 ± 0.9	5.2 ± 1.7	4.0 ± 2.7	NA	NA
Pouch duration (yr)	NA	NA	NA	NA	NA	2.8 ± 1.5	3.9 ± 2.2
BMI (kg/m^2)	24.5 ± 2.3	25.2 ± 1.6	24.8 ± 3.5	23.9 ± 2.7	24.4 ± 3.1	24.9 ± 2.2	24.3 ± 3.5
Mayo score	NA	≤ 2	4.2 ± 0.7	8.5 ± 1.2	11.5 ± 0.3	≥ 7	< 7
Age at colectomy	NA	NA	NA	NA	NA	40.6 ± 12.9	42.4 ± 9.22
Standard medication (%)	NA	0 (0)	5 (46.0)	8 (80.0)	10 (100)	8 (100)	3 (27.0)
Smoking (% at recruitment)	10 (62.5)	4 (40.0)	3 (27.3)	2 (20.0)	3 (30.0)	4 (50.0)	5 (45.5)
Previous number of episodes of pouchitis (%)	NA	NA	NA	NA	NA	4 (50.0)	3 (27.0)
Number of patients with chronic pouchitis (%)	NA	NA	NA	NA	NA	3 (37.5)	2 (18.0)
Secondary causes of pouchitis (%)	NA	NA	NA	NA	NA	2 (25.0)	2 (19.0)

UC: Ulcerative colitis; NA: Not available; Pouch: Ileal pouch established during ileal pouch-anal anastomosis surgery; pouchitis: Inflammation of ileal pouch.

Beijing, China) was used to isolate DNA from frozen feces individually, following the manufacturer's guidelines and as previously described[14]. Following 1% agarose gel electrophoresis, the eluted DNA was quantified on a NanoDrop 2000 Spectrophotometer.

PCR amplification

The genomic DNA and universal primers including forward and reverse primers (AuGCT DNA technologies, Beijing, China) were employed to amplify the whole fragment V3 region of bacterial 16S rRNA gene.

After 15 cycles of thermocycling on a PCR system (Bio-Rad, Hercules, CA, United States) the amplified product was verified by 2% agarose electrophoresis. The amplified DNA was quantified on a NanoDrop 2000 Spectrophotometer, and recorded by a DH2000 gel imaging analysis.

DGGE for amplified 16S rRNA gene

DGGE was chosen to separate PCR amplicons according to the rules of Muyzer *et al*[15], with some modifications. A 10% polyacrylamide combined with Tris-acetate-EDTA (TAE) buffer was used for polyacrylamide gels with a denaturing gradient ranging from 30% to 70%. A stacking gel was added before polymerization of the denaturing gel, followed by appropriate comb insertion. Electrophoresis was performed at 200 V for 5 min and 85 V for 16 h in 0.5 × TAE buffer subsequently at a constant 60 ℃. After staining with $AgNO_3$[16], the gels were desiccated overnight at 60 ℃.

Digital processing of DGGE profiles

Following the manufacturer's instructions, DGGE profiles were analyzed digitally using Quantity One-4.6.5 in the UNIVERSAL HOOD Ⅱ Gel Imaging System (Beijing, China). After normalizing the gels according to the results for the healthy controls, the band in each sample was marked by the software, and manual corrections were conducted. The number of DGGE bands was shown as the mean ± SD. Based on the gray value, Dice similarity and UPGMA tree analyses were conducted using Quantity One software. Canoco software was used to conduct principal component analysis (PCA).

DGGE band extraction and sequencing

Based on the digital results, the bands distinguishing the groups were excised from the gel, purified and sequenced. The gel slice, in 15 μL of TE buffer, was heated at 65 ℃ for 10 min to elute the DNA from the gel. The DNA solution was amplified using the universal V3 primers F357+ a GC clamp and R518. DGGE gel expansion facilitated the purification of the bands. DGGE with an adjusted gradient of 32 was used to check the amplicons, which were excised at least three times until a single band was obtained. The DGGE was repeated to purify the PCR product before sequencing. In the final round, the amplicons were analyzed with the original sample profiles from which they were excised and analyzed visually for purification of the correct bands.

When the purified bands matched with the targeted bands, the amplicons sequenced using an ABI Prism system and primers R518 and F357 (without the clamp). BioNumerics software was used to analyze the sequences. BLAST homology searches were performed against the GenBank DNA database. According to BLAST results, the sequences of phylogenetic neighbor species, whose similarities were up to 90%, were included for reference in the cluster analysis using multiple sequence alignments. The purified band sequences were allocated to the most probable species according to the average linking method.

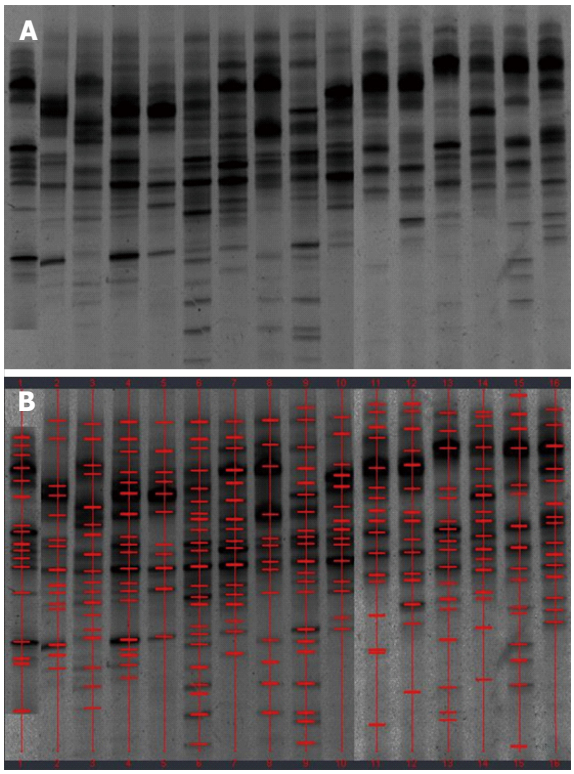

Figure 1 Denaturing gradient gel electrophoresis profiles of fecal samples from healthy controls. A: Denaturing gradient gel electrophoresis (DGGE) profiles; B: Marked DGGE profiles. DGGE bands showed relative stability among different individuals.

Statistical analysis

Quantity One software was used for Dice similarity analysis and UPGMA tree analysis. DGGE strips data are presented as mean ± SD. The Shapiro-Wilk test was used to test the normality of the band number and Dice analysis data. The content of each sample was similar. The homogeneity of variance was robust and highly efficient. The band number of DGGE strips showed a normal distribution, unlike the Dice analysis results. Therefore, the three groups were tested using a Student-Newman-Keuls test. The Bonferroni test was chosen to compare DGGE strips and band numbers among the five groups. An extension *t* test after non-parametric Kruskal-Wallis *H* test was employed to compare the Dice analysis results among multiple groups. The correlation between disease activity and bacterial count was assessed using the Spearman correlation coefficient. SPSS 19.0 software was employed to analyze all the data. Two-tailed tests were used in all analysis and *P* values of $\leqslant$ 0.05 were considered statistically significant.

RESULTS

Bacteria in fecal samples from UC patients

The demographic details of the study patients are shown in Table 1. DNA extracts from the fecal samples from different individuals presented variable number of bands after PCR-DGGE analysis. A band representing identical or similar sequences of the V3 regions of the 16S rRNA gene was observed, reflecting the dominant bacterial communities in the fecal samples.

Examination of digital DGGE profiles from healthy controls showed relative stability among different individuals (Figure 1). Profiles from UC patients shown in Figure 2 suggested significant variation in the position and number of bands compared with the healthy controls. The number of bands, which reflected the diverse microbiota, was 17 ± 3 in the 16 healthy controls and 13 ± 3 in the 41 UC patients ($P = 0.001$). Differences were also seen among the subgroups of UC patients (Figure 3). These results reveal that the number of predominant microbiota was negatively correlated with the Mayo classification ($r = -6.591$, $P < 0.01$). The Kruskal-Wallis *H* test showed greater similarity between groups than within the groups, which revealed variation in the predominant microbiota with clinical status (Table 2). UPGMA tree analysis showed similar results (Figure 4). PCA analysis of healthy and UC groups revealed large differences in the predominant species among the control group, remission and mild group, and the moderate and severe group (Figure 5).

Sequencing results after purification (based on digital DGGE profiles) showed the presence of a greater number of *C. perfringens* and fewer *Faecalibacterium prausnitzii* (*F. prausnitzii*) and *Eubacterium rectale* (*E. rectal*) in UC group compared with the control group. *C. perfringens* was present predominantly in severe UC.

Bacteria in fecal samples of pouchitis patients

Significant changes occurred in the position and number of bands from patients with pouchitis when compared with NP and healthy controls (Figure 6). Differences in the number of bands in the controls (17 ± 3 bands), NP (11 ± 3 bands) and pouchitis (8 ± 2 bands) are shown in Figure 7 (ANOVA test). A Bonferroni test showed greater similarity between groups than within groups, suggesting differences in the predominant species in the healthy controls, NP and pouchitis groups (Table 3). These results suggested that patients with pouchitis had an altered microbiota composition compared with healthy individuals. UPGMA tree analysis showed similar results (Figure 8). PCA analysis of healthy control and pouchitis groups revealed great variation in the predominant species in pouchitis compared with non-pouchitis and healthy controls, which also differed from each other (Figure 9).

Sequencing results after purification (based on digital DGGE profiles) showed fewer *E. rectale* and more *C. perfringens* in the pouchitis group compared with the NP and control groups.

As shown in Table 4, the DGGE profiles in pouchitis patients varied significantly from UC in remission to the severe state, while the NP group of patients differed from UC in remission. The results showed

Table 2 Dice analysis of healthy control and ulcerative colitis subgroups

Group	Control	Remission	Mild	Moderate	Severe
Control	(49.79 ± 11.24)%[a]	(35.32 ± 14.86)%	(30.13 ± 11.23)%	(31.98 ± 16.48)%	(28.18 ± 14.99)%
Remission		(42.89 ± 18.29)%[a]	(32.79 ± 13.68)%	(30.22 ± 15.28)%	(26.28 ± 13.94)%
Mild			(41.83 ± 16.38)%[a]	(29.89 ± 13.10)%	(28.31 ± 18.39)%
Moderate				(43.45 ± 21.32)%[a]	(28.88 ± 13.69)%
Severe					(37.12 ± 19.98)%[a]

[a]$P < 0.05$; result from similarity in the same group *vs* similarity in different groups.

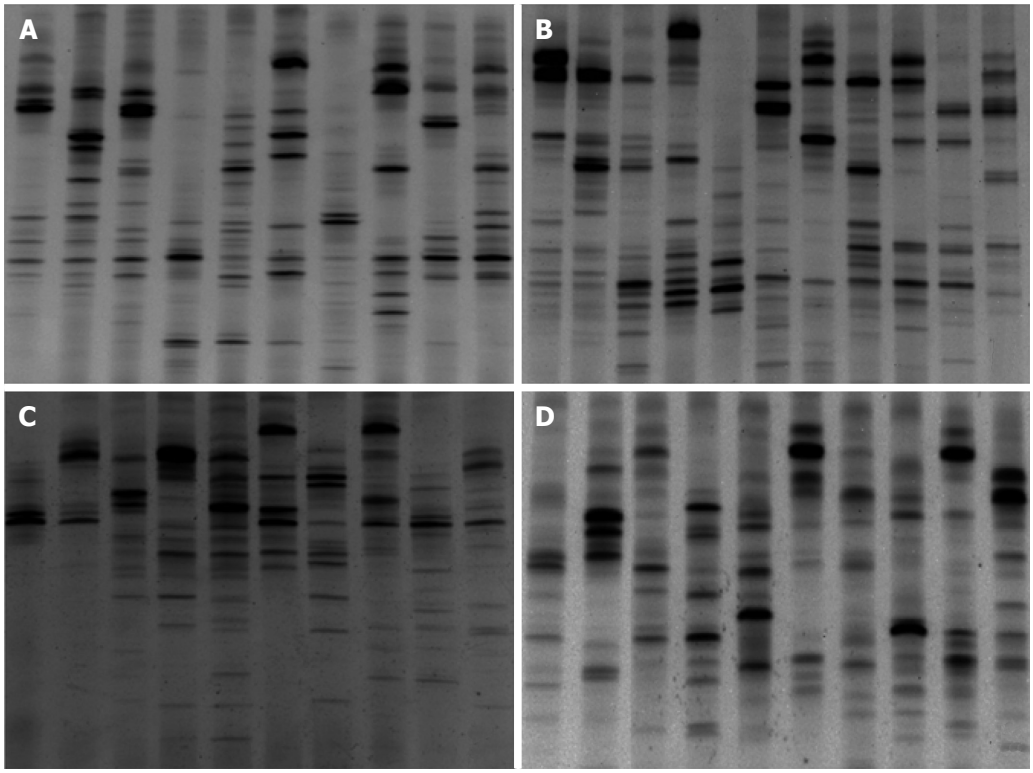

Figure 2 Denaturing gradient gel electrophoresis profiles showed microbial biodiversity in different ulcerative colitis groups. A: Ulcerative colitis (UC) patients in remission; B: UC patients in the mildly active stage; C: UC patients in the moderately active stage; D: UC patients in the severely active stage.

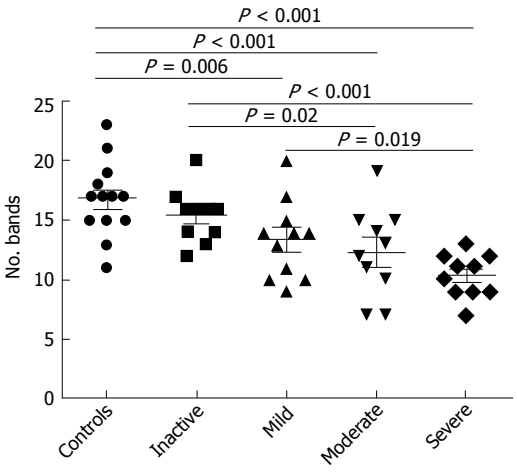

Figure 3 Number of bands in denaturing gradient gel electrophoresis profiles of samples obtained from 41 ulcerative colitis patients. The number of bands decreased significantly from healthy controls to severe ulcerative colitis.

Table 3 Dice analysis of healthy control and pouchitis subgroups

Group	Control	Pouchitis	NP
Control	(49.79 ± 11.24)%[a]	(25.33 ± 11.13)%	(28.86 ± 14.23)%
Pouchitis		(35.43 ± 13.30)%[a]	(20.87 ± 12.31)%
NP			(35.39 ± 10.80)%[a]

[a]$P < 0.05$; result from similarity in the same group *vs* similarity in different groups. NP: No pouchitis.

that patients with a pouch have an altered microbiota diversity compared with UC patients (Figure 10). The diversity of the microbiota from pouchitis patients was lower than that in severe UC patients. A normal pouch can be can be present along with mild, moderate and severe UC. The sequencing results for the UC and pouchitis groups showed that they shared a similar microbiota, such as *C. perfringens*.

Table 4 Bacterial diversity comparison

	Healthy controls	UC				Pouch	
		Remission	Mild	Moderate	Severe	NP	Pouchitis
Healthy controls	-	0.298	0.006[a]	0.001[a]	0.000[a]	0.000[a]	0.000[a]
Remission UC	0.298	-	0.113	0.020[a]	0.000[a]	0.014[a]	0.003[a]
Mild UC	0.006[a]	0.113	-	0.404	0.019[a]	0.007[a]	0.125
Moderate UC	0.001[a]	0.020[a]	0.404	-	0.128	0.009[a]	0.448
Severe UC	0.000[a]	0.000[a]	0.019[a]	0.128	-	0.019[a]	0.496
NP: PDAI < 7	0.000[a]	0.014[a]	0.007[a]	0.009[a]	0.019[a]	-	0.034[a]
Pouchitis: PDAI ⩾ 7	0.000[a]	0.003[a]	0.005[a]	0.448	0.496	0.034[a]	-

[a]$P < 0.05$. NP: No pouchitis; UC: Ulcerative colitis; Pouch: Ileal pouch established during ileal pouch-anal anastomosis surgery; pouchitis: Inflammation of ileal pouch; PDAI: Pouchitis Disease Activity Index.

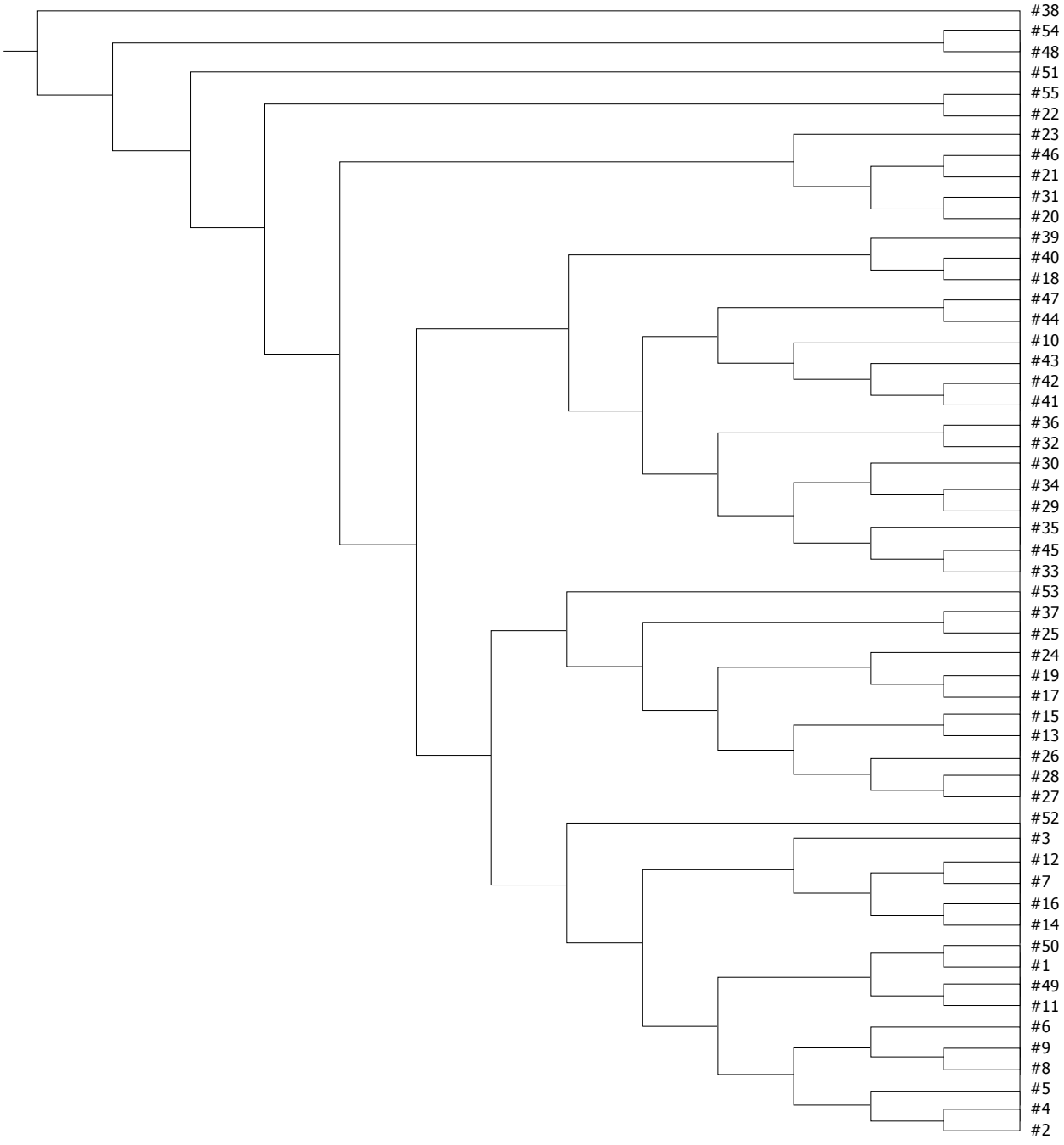

Figure 4 UPGMA tree analysis of healthy controls and ulcerative colitis patients at different stages. 1-16: Healthy controls; 17-26: Ulcerative colitis (UC) patients in remission; 27-37: UC patients in the mildly active stage; 38-47: UC patients in the moderately active stage; 48-57: UC patients in the severely active stage. UPGMA tree analysis showed a significant difference among groups of healthy controls and UC patients at different stages.

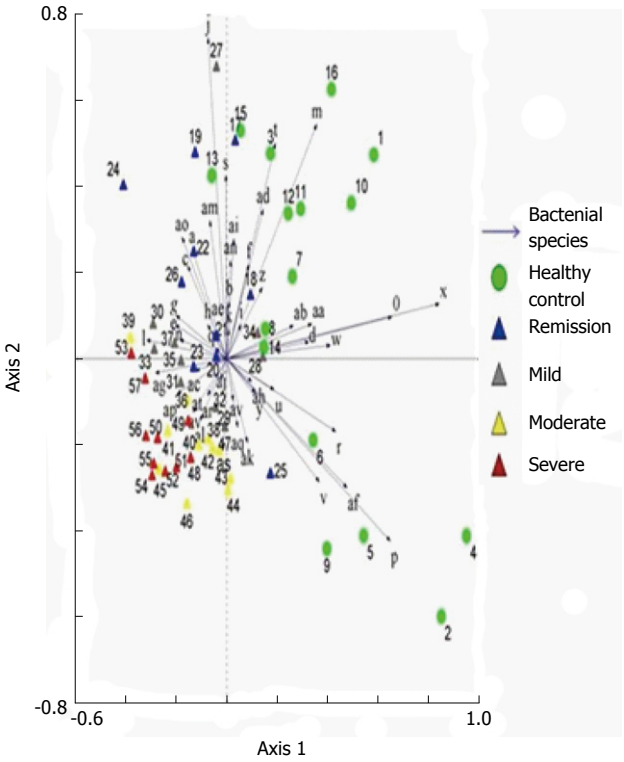

Figure 5 Principal component analysis of denaturing gradient gel electrophoresis microbial profiles in fecal samples of healthy controls and ulcerative colitis patients at different stages. Clustering of similar microbial profiles showed systematic differences among different groups.

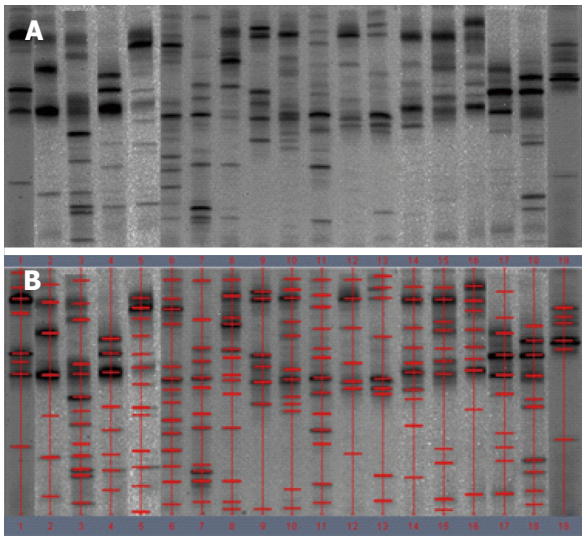

Figure 6 Denaturing gradient gel electrophoresis profiles of fecal samples from patients with pouchitis. A: Denaturing gradient gel electrophoresis (DGGE) profiles; B: Marked DGGE profiles. DGGE bands revealed the relative stability of the microbiota in pouchitis group.

DISCUSSION

In this study, we focused on UC patients after IPAA surgery, and specifically compared patients developing pouch inflammation with those without surgery. Our digital analysis of stool samples showed that the predominant microbiota in UC patients was

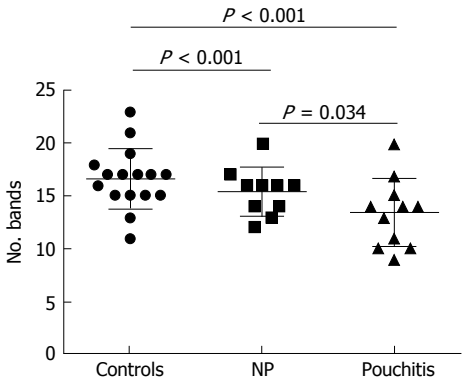

Figure 7 Number of bands in denaturing gradient gel electrophoresis profiles of samples obtained from patients receiving surgery. The number of bands was reduced significantly in pouchitis compared with the control group and the no pouchitis (NP) group.

reduced compared with the healthy group. Sequence analysis showed more *C. perfringens* and less *F. prausnitzii* and *E. rectale* in the UC group. Levels of *E. rectale* (a butyrate-producing bacteria) were significantly reduced on UC mucosa[17], and had high age dependence. High clinical activity indices, as well as sigmoidoscopy scores, were associated with *E. rectale*[18]. Vermeiren demonstrated fewer *E. rectale* shown in UC patients *via* a dynamic gut model of the mucin environment[17]. *C. perfringens*, a Gram-positive, anaerobic, spore-forming bacillus, is found in the intestinal contents of both animals and humans[19]. *C. perfringens* is an intestinal commensal organism as well as a pathogen, for example, *via* production of toxins that damage the host tissues[20]. *C. perfringens* exerts proteolytic and mucinase activity, both of which could mediate the pathogenesis of inflammatory bowel disease (IBD)[21]. *C. perfringens* found in IBD patients, which is thought to be an important factor during the immunopathogenesis of IBD, could result from dysbiosis[22]. Falk showed that there were more *C. perfringens* in pouchitis patients[24]. Another study found that 21% of the total bacteria in colonic specimens collected from patients with UC belonged to clostridia of clusters Ⅰ, Ⅱ and Ⅺ, which were not found in the control groups[23]. We should keep in mind that ileum tissues of UC patients were the origin of the present pouches. *F. prausnitzii* is the most host species-specific microbe in the study of IBD. Sokol *et al*[25] studied a small group of 17 UC patients and reported a reduction in *F. prausnitzii* in active UC patients. A strong anti-inflammatory effect of *F. prausnitzii* has been demonstrated both *in vitro* and *in vivo*[26]. Machiels *et al*[27] observed a significant inverse correlation between disease activity and numbers of *F. prausnitzii*, indicating that a deficiency of this species provokes or enhance inflammation. *F. prausnitzii* produces high concentrations of butyrate, a vital energy source for colonocytes, which also prevents mucosal atrophy. Consequently, butyrate improves the mucosal barrier function of the colon. Furthermore, butyrate

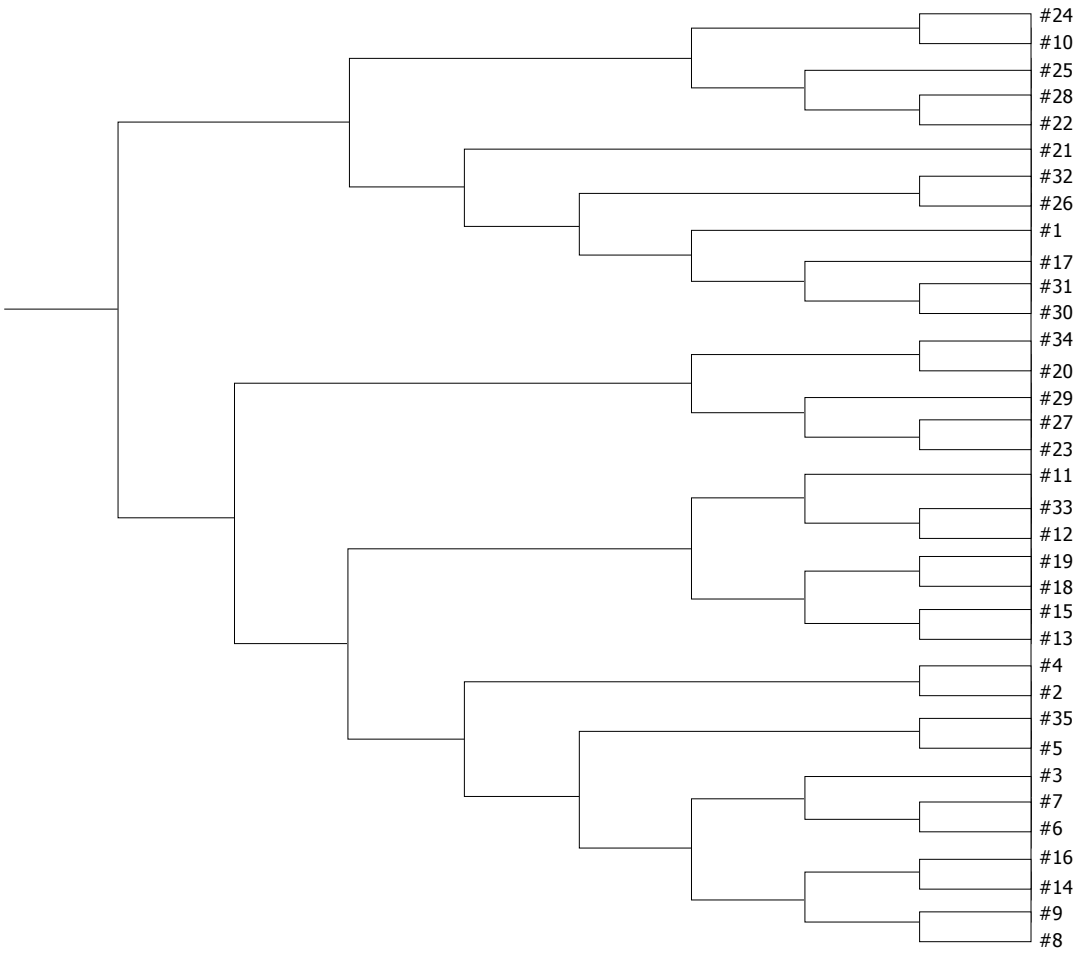

Figure 8 UPGMA tree analysis of healthy controls and postoperative patients. 1-16: Healthy controls; 17-27: UC patients without pouchitis after IPAA; 28-35: Patients with pouchitis. UPGMA tree analysis showed a significant difference among the three groups.

exhibits immunomodulatory and anti-inflammatory effects by downregulating pro-inflammatory cytokines[28]. Our data showed that bacterial biodiversity in feces decreased distinctly with the severity of Mayo classification compared with healthy controls. Studies have demonstrated that the mucosal biopsies from patients with active Crohn's disease (CD) or active UC showed reduced bacterial diversity after analysis of 16S rRNA genes[29]. Furthermore, Manichanh *et al*[30] reported a reduction in the phylum Firmicutes in CD in remission using an extensive metagenomic analysis. Consistent with previous studies, our results confirmed that bacterial diversity was reduced in fecal samples from UC patients at different grades, and demonstrated changes in the microbial composition among subgroups in UC. The decreased biodiversity in UC might disrupt the stability of gut ecosystem. The results revealed that changes in the predominant bacteria were consistent with the Mayo classification. Therefore, we suggest that the fecal microflora in UC patients is reduced in aggravated intestinal lesions. A previous study by Wills *et al*[31] reported patient-specific shifts in microbial composition in UC patients showing altered pathological activity over time. The changes were more pronounced in CD cases than in UC patients, suggesting their role in the inflammatory process of UC.

By contrast, the number of bands on the DGGE profiles from pouchitis patients varied between UC and healthy controls. We showed a decrease in bacterial diversity and reduced abundance of predominant bacteria in UC pouches. *R. gnavus* infection, especially occurring as the predominant microbiota before colectomy, was shown to increase the risk of pouchitis 1 year after IPAA[12]. *R. gnavus* produces the bacteriocin ruminococcin A, which inhibits the growth of phylogenetically-related species and various bifidobacterial and clostridial species[32]. Ruminococcin A also degrades intestinal mucin[33] and induced α-galactosidase and β-glucuronidase activity *in vitro*[34]. β-glucuronidase activity generates toxic metabolites in the colon, which provoke local inflammation. Png *et al*[35] observed an increase in mucolytic bacteria, including *R. gnavus*, in biopsies of patients with UC and CD. Our data are supported by reports from several groups that analyzed fecal or biopsy samples using different DNA-based methods[7], further confirming the association between changes in microbiota and pouchitis. By contrast, the variability in endogenous factors, including secretion of mucins,

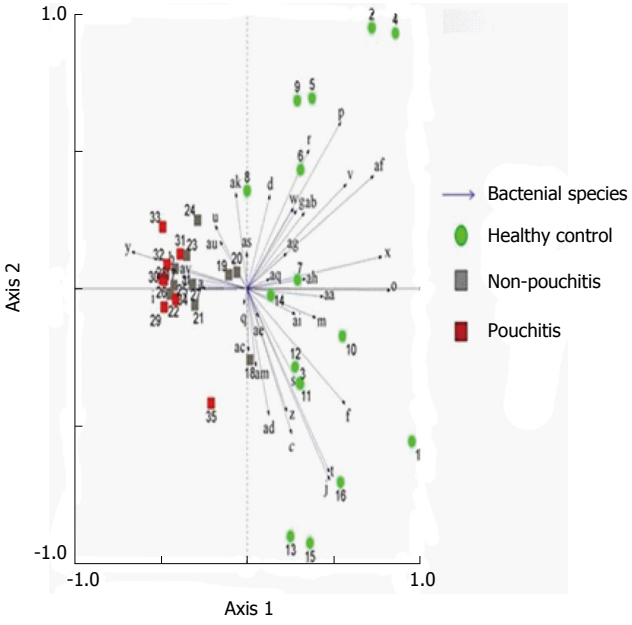

Figure 9 Principal component analysis of denaturing gradient gel electrophoresis microbial profiles in fecal samples of healthy controls and patients with pouch (with or without pouchitis). Clustering of similar microbial profiles showed significant differences among the three groups.

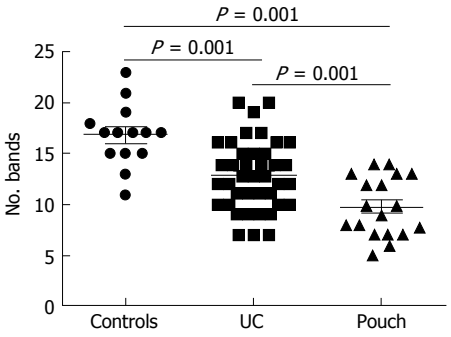

Figure 10 Number of bands in denaturing gradient gel electrophoresis profiles of samples obtained from all subjects. The number of bands was reduced significantly in pouch group (UC patients who underwent IPAA, with or without pouchitis) compared with the control group and the UC group. UC: Ulcerative colitis.

defensins, cytokines and immunoglobulins, might also affect the composition of predominant bacterial species in UC and pouchitis. However, data about the effects of these secretions effects on the variability of UC is limited. Studies involving UC have revealed that a high percentage of fecal bacteria (about 30%-40% of dominant species) belong to unusual genera in healthy populations[36]. Hypothetically, the decreased number of stable commensals in individuals that are genetically susceptible to pouchitis would break this first line of natural defense against potentially invasive bacteria, resulting in inflammation.

In conclusion, our research demonstrated reduced biodiversity of fecal microbiota in UC and pouchitis patients. There were fewer *F. prausnitzii* and *E. rectale* in UC, more *R. gnavus* in pouchitis and more *C. perfringens* in both UC and pouchitis.

COMMENTS

Background

Pouchitis is a commonly-seen complication in patients with ulcerative colitis (UC) following ileal pouch-anal anastomosis (IPAA) procedure. The gut microbiome is considered to play a vital role in the occurrence and development of UC. In addition, antibiotics and probiotics have already been used to treat and prevent pouchitis. However, direct evidence of dysbacteriosis in pouchitis is lacking.

Research frontiers

Gut microbiota is hot topic in field of intestinal inflammation, especially in inflammatory bowel disease (IBD), where data are often conflicting.

Innovations and breakthroughs

The authors demonstrated the role of the reduced diversity and the changed composition of intestinal microbiota in the pathogenesis of pouchitis. Reduced levels of *Faecalibacterium prausnitzii* and *Eubacterium rectal* were confirmed in UC patients, and *Clostridium perfringens* was significantly increased in UC and pouchitis.

Applications

These findings provide new clues to better understand the pathogenesis of pouchitis in UC patients subjected to IPAA, although the findings will not be used immediately in the treatment of pouchitis.

Terminology

Pouchitis: An inflammation in the intestinal pouch, which is established following the proctocolectomy IPAA procedure for patients with UC to prevent permanent abdominal fistula. Pouchitis could have serious consequences, such as bloody diarrhea and even ileal pouch failure.

Peer-review

The manuscript definitely deals with a hot topic in the IBD and intestinal inflammation field, where data are often conflicting. The deep insight and detailed description made by the authors of the diversity of microbiota in healthy people and patients will provide new clues to better understand the pathogenesis of pouchitis following IPAA for UC.

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P- Reviewer: Caviglia R, Pastorelli L **S- Editor**: Yu J
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World Journal of Gastroenterology

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DOI: 10.3748/wjg.v22.i40.8940

World J Gastroenterol 2016 October 28; 22(40): 8940-8948
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

ORIGINAL ARTICLE

Retrospective Study

Ansa pancreatica as a predisposing factor for recurrent acute pancreatitis

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Author contributions: Hayashi YT and Gonoi W designed the research, analyzed data, and drafted the manuscript; Yoshikawa T, Hayashi N and Ohtomo K revised the manuscript; all authors have read and approved the final version to be published.

Supported by JSPS KAKENHI, No. 25870148.

Institutional review board statement: Based on the Declaration of Helsinki, the Research Ethics Committee of the University of Tokyo Hospital approved the prospective and retrospective use of the clinical, biochemical, and radiographic data for the present cross-sectional study.

Informed consent statement: Our institutional review board approved waiver of informed consent for the present cross-sectional study about the patient (pancreatitis) group. All of the subjects of community group provided written informed consent for the comprehensive epidemiological study.

Conflict-of-interest statement: None.

Data sharing statement: No additional data are available.

Manuscript source: Invited manuscript

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Received: June 14, 2016
Peer-review started: June 17, 2016
First decision: July 12, 2016
Revised: August 11, 2016
Accepted: September 14, 2016
Article in press: September 14, 2016
Published online: October 28, 2016

Abstract

AIM

To determine the non-biased prevalence and clinical significance of ansa pancreatica in patients with acute pancreatitis using magnetic resonance imaging (MRI).

METHODS

Our institutional review board approved this cross-sectional study, which consisted of a community-based cohort of 587 consecutive participants in a whole-body health-check program, and 73 subjects with episode of acute pancreatitis (55 patients with a single episode of acute pancreatitis, and 18 patients with recurrent acute pancreatitis). All of the subjects underwent abdominal MRI including magnetic resonance cholangiopancreatography, medical examinations, and blood tests. Two board-certified, diagnostic, abdominal radiologists evaluated the images, and ansa pancreatica was diagnosed based on its characteristic anatomy on MRI.

RESULTS

Compared with the community group [5/587 (0.85%)], patients with recurrent acute pancreatitis had a significantly higher frequency of ansa pancreatica [2/18

(11.1%)] (P = 0.016; OR = 14.3; 95%CI: 1.27-96.1), but not compared with patients with single-episode acute pancreatitis [1/55 (1.8%)] (P = 0.42; OR = 2.1; 95%CI: 0.44-19.7). Multiple logistic regression analysis using age, alcohol intake, presence of ansa pancreatica, and presence of autoimmune disease as independent covariates, revealed a significant relationship between the presence of ansa pancreatica and recurrent acute pancreatitis. The presence of autoimmune disease was also significantly associated with the onset of recurrent acute pancreatitis. On the other hand, neither age nor alcohol intake were significantly related to the onset of recurrent acute pancreatitis.

CONCLUSION

The present study is the first to provide robust evidence that the presence of ansa pancreatica is significantly associated with recurrent acute pancreatitis.

Key words: Anatomy; Cholangiopancreatography; Pancreatitis; Magnetic resonance imaging; Pancreatic duct

Core tip: Ansa pancreatica is a rare anatomical variation of the accessory pancreatic duct and was hypothesized to be a predisposing factor for pancreatitis. However its *in vivo* prevalence was unknown and no case-control study has confirmed its clinical significance. This study is the first case-control study to determine the non-biased prevalence and provide robust evidence that the presence of ansa pancreatica is significantly associated with recurrent acute pancreatitis, using non-invasive magnetic resonance imaging.

Hayashi TY, Gonoi W, Yoshikawa T, Hayashi N, Ohtomo K. Ansa pancreatica as a predisposing factor for recurrent acute pancreatitis. *World J Gastroenterol* 2016; 22(40): 8940-8948 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i40/8940.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i40.8940

INTRODUCTION

Pancreatitis is a severe inflammatory disease that is critical in some patients. Furthermore, serious damage of pancreatic tissue leads to dysfunction of the endocrine and exocrine systems, particularly in the chronic phases of the disease. There are several known causes of pancreatitis, including excessive alcohol consumption, biliary stones, trauma, autoimmunity, metabolic disorders, drugs, iatrogenic, infection, genetic mutations, malignancy, and heredity factors[1-3]; and morphological aberrations such as atypical arrangement of the pancreaticobiliary ductal system[4,5], pancreas divisum[5-8], or a meandering main pancreatic duct[9]. However, recurrent episodes of acute pancreatitis is idiopathic in as many as 20% of patients[8,10,11].

Dawson and Langman[12] first reported ansa pancreatica in 1961. In this pancreatic duct variant, the accessory duct is obliterated at its junction with the ventral duct, and is replaced with an additional curved communicating duct between the ventral and dorsal ducts at the pancreatic head. This additional duct arises from the ventral duct, runs into the caudal side of the ventral duct, turns to the ventral side with a reversed S-shaped curve, and finally terminates in and around the minor papilla (Figures 1 and 2)[4,12-14].

In several case reports, ansa pancreatica was hypothesized to be a predisposing factor for pancreatitis, particularly acute pancreatitis[14,15]. To date, however, no case-control study has confirmed its clinical significance. Therefore, the present study aimed to investigate the relationship between ansa pancreatica and acute pancreatitis.

MATERIALS AND METHODS

Ethics

This study conformed with the Declaration of Helsinki. The prospective and retrospective use of the clinical, biochemical, and radiographic data was approved by the Research Ethics Committee of the University of Tokyo Hospital for the present cross-sectional study.

Subjects

The subjects were divided into a community group (group 1) and patients with acute pancreatitis (group 2); the latter group was divided into two subgroups. Group 1 included consecutive community residents who attended a paid health checkup program between October 12, 2006 and May 31, 2007 that was advertised *via* leaflets and the Internet. The program included blood testing; evaluation of drinking and smoking habits; a thorough medical and subjective symptom history; and a physical examination performed by a physician. Additionally, whole-body imaging studies were performed, and included magnetic resonance imaging (MRI) of the abdomen and magnetic resonance cholangiopancreatography (MRCP). Laboratory blood testing comprised WBC and platelet counts, hemoglobin, glycated hemoglobin, amylase, glucose, insulin, C-reactive protein, aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, γ-glutamyltransferase, total bilirubin, and high- and low-density lipoprotein. All of the tests in individual subjects were performed on the same day. All of the subjects provided written informed consent for the comprehensive epidemiological study. Only subjects who underwent all of the examinations listed above were included in the study.

Group 2 included patients who were diagnosed with acute-type pancreatitis regardless of the cause. These patients were divided into two subgroups depending

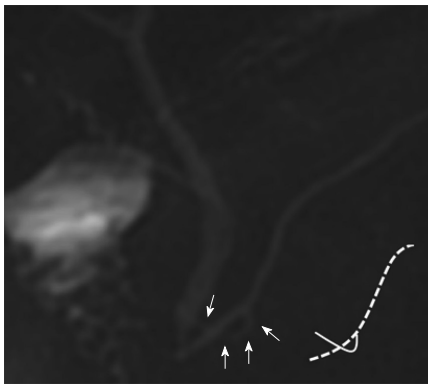

Figure 1 Ansa pancreatica depicted on magnetic resonance images and its schematic image. Magnetic resonance cholangiopancreatography reveals the absence of a normal type of accessory pancreatic duct and the presence of an additional curved duct in the head of pancreas (white arrows). The additional duct is seen crossing the main pancreatic duct on this projection image. Schematic image of the pancreatic duct is described on the right side. The broken line indicates the ventral duct and the solid black line represents ansa pancreatica.

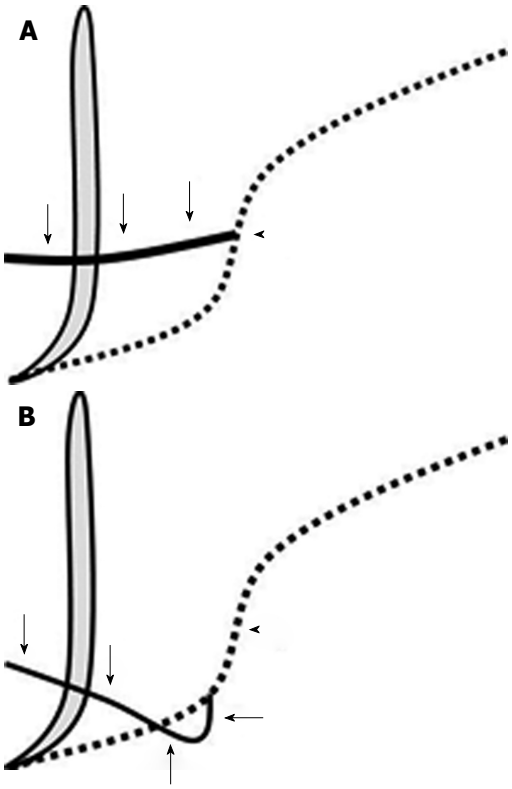

Figure 2 Schematic images of a normal pancreatic duct (A) and ansa pancreatica (B). The vertical thick gray line indicates the common bile duct and the broken lines indicate the ventral duct. The solid black line represents the normal accessory duct in (A) and ansa pancreatica in (B). In the normal type (A), the accessory duct (arrows) arises near the flexion point (arrowhead) of the main pancreatic duct and runs towards the minor papilla horizontally and to the right. In ansa pancreatica (B), the additional duct (arrows) arises from the caudal side of the flexion point (arrowhead) of the main pancreatic duct. It runs caudally at first, then rightward and ventrally, crossing the ventral duct, and finally terminates near the minor papilla.

on whether they had single-episode acute pancreatitis or recurrent acute pancreatitis. We performed a retrospective medical chart review of all consecutive patients who were suspected of having single-episode or recurrent acute pancreatitis and who underwent abdominal MRI and MRCP between January 1, 2003 and October 17, 2013. To determine the type of pancreatitis, the entire medical records for all patients were reviewed to evaluate the type of onset and the cause of pancreatitis. The respective diagnoses were assessed using the latest criteria available as of March 2014. Single-episode acute pancreatitis was defined used the Japanese (JPN) Guidelines for the management of acute pancreatitis[16]. Recurrent acute pancreatitis was defined as the following: $\geqslant$ 2 well-documented episodes of abdominal pain typical of acute pancreatitis that were separated by > 2 mo; and $\geqslant$ 1 of (1) lipase or serum amylase elevation > 3 × the upper limit of normal, and/or (2) features consistent with acute pancreatitis on diagnostic imaging[11,17].

The cause of pancreatitis was also examined in the acute pancreatitis group. Idiopathic pancreatitis was diagnosed after excluding all established causes of pancreatitis, and according to the results of physical examination, imaging, and biochemical tests. Genetic testing and manometric assessments were conducted only if deemed necessary.

The exclusion criteria were as follows: incomplete clinical evaluations, pancreatitis with chronic onset (diagnosed using the latest criteria of the revised Japanese clinical diagnostic criteria for chronic pancreatitis)[18], the quality of MRI scans was inadequate to evaluate the accessory duct (we used stricter criteria compared with the usual clinical standard), post-pancreaticoduodenectomy status, and the presence of a pancreatic or biliary tumor occupying the head of pancreas. The region (undetectable, head, body, tail, or $\geqslant$ 2 of these pancreatic regions) and severity of inflammation were also assessed using the Japanese Ministry of Health, Labour, and Welfare severity scoring system for acute pancreatitis (JPN score 2008)[19].

Our institutional review board approved waiver of informed consent for the present cross-sectional study about the pancreatitis group.

MRI technique

For the community group, MRI was performed using 3 T scanners (GE Medical Systems, Waukesha, WI, United States). During breath hold, heavily T2-weighted MRCP images were acquired in the coronal plane using a two-dimensional (2D) half-Fourier fast spin echo (FSE) technique [repetition time/echo time (TR/TE), ∞/600 ms; slice thickness (ST), 40 mm]. Four coronal and oblique-coronal projection images were reconstructed. Transaxial FSE T2-weighted images [TR/TE, ∞/80 ms; ST, 3 mm (without gap)] and fat-suppressed T1-weighted images were also acquired for complementary interpretation, using a three-dimensional (3D) gradient echo technique [TR/TE, 3.5/1.5 ms; flip angle, 15°; ST, 3 mm (with 1.5

mm overlap)]. Subjects were not premedicated.

For the acute pancreatitis group, MRI was performed using either 3 T scanners (GE Medical Systems) or 1.5 T scanners (GE Medical Systems; Siemens AG, Erlangen, Germany; and Toshiba Medical Systems, Tochigi, Japan). With the patients in breath hold, heavily T2-weighted MRCP images were acquired by 2D half-Fourier FSE (TR/TE, 2400-∞/600-1100 ms; ST, 30-50 mm) and respiratory-gated 3D half-Fourier FSE [TR/TE, 1300-∞/500-900 ms; ST, 1.2-2.0 mm (without gap)]. Coronal and oblique-coronal projection images were reconstructed. We also acquired transaxial and coronal T2-weighted images [FSE; TR/TE, 1300-∞/80-150 ms; ST, 5 mm (without gap)] and fat-suppressed T1-weighted images [3D gradient echo; TR/TE, 3-840/1.5-140 ms; flip angle, 15°; ST, 1.5 mm (without gap)] for complementary interpretation. Before MRI, the patients were administered manganese chloride solution (Bothdel Oral Solution 10; Kyowa Hakko Kirin, Tokyo, Japan) as negative oral contrast agent.

Image interpretation

All MRI scans were reviewed by two board-certified, qualified (6-8 years' experience of pancreaticobiliary imaging), diagnostic, abdominal radiologists on picture archiving and communication system workstations (Centricity; GE Medical Systems). Both radiologists were blinded to the clinical information. One radiologist acted as the main interpreter and other supervised the image interpretation.

Pancreatic ductal anatomy was evaluated on MRI scans as follows. Ansa pancreatica was considered present if the oblique-coronal MRCP plane showed (1) the upstream accessory duct was obliterated; and (2) the additional duct arose from the ventral duct, ran caudally, then dextrad and ventrally, and finally terminated near the minor papilla. The radiologist was asked to state whether ansa pancreatica was present or not. All radiographic findings related to the pancreaticobiliary system were recorded (*e.g.*, other variants of pancreatic ductal fusion, pancreatic ductal/ductile dilation or irregularity, gallstones, pancreatic cystic lesions, cystic polyps, pancreatic parenchymal atrophy, biliary morphological defects, adenomyomatosis, and juxtapapillary duodenal diverticulum). In suspected cases of ansa pancreatica, the two radiologists evaluated the images to reach a consensus on its presence or absence. When the opinions disagreed, the supervisor's interpretation took precedence.

Statistical analysis

For univariate comparisons between groups, Welch's *t* test was used for continuous variables and, for categorical values, Fisher's exact test was used; 0.05 was set as the level of statistical significance. Bonferroni's method was used to correct family-wise error. Multiple logistic regression analysis was used to identify factors that were associated with pancreatitis. To prevent overestimation of the number of predictive values, variables with $P < 0.05$ in the univariate analyses were selected before applying family-wise error correction. All statistical computations were performed using R Ver. 2.9 (free software; The R Foundation for Statistical Computing, Vienna, Austria; http://cran.r-project.org/).

RESULTS

Subjects

In group 1674 subjects completed the study. Subjects were excluded because of post-pancreaticoduodenectomy (n = 1); incomplete MRI scans (n = 1); intraductal papillary mucinous neoplasm in the head of pancreas (n = 9); and inadequate image quality to evaluate the accessory duct, most commonly due to hindered visualization of the pancreatic ducts owing to artifact from gastrointestinal signal (n = 76). The final evaluable cohort of 587 community subjects included 250 women (mean age, 57.0 years; range, 31-84 years) and 337 men (mean age, 56.6 years; range, 40-86 years) (Table 1). The majority of subjects were Japanese (one subject was Korean). None of these subjects complained of pancreatic pain. Six subjects had a history of pancreatitis, of which four had acute pancreatitis and two had chronic pancreatitis. The medical records for the four subjects with acute pancreatitis were unavailable so we could not determine whether they had recurrent acute pancreatitis or single-episode acute pancreatitis.

In group 2, a total of 6103 MRCP scans were performed between January 1, 2003 and October 17, 2013. After excluding overlapping subjects and patients without single-episode acute pancreatitis or recurrent acute pancreatitis, 102 patients remained, of which 78 had single-episode acute pancreatitis and 24 had recurrent acute pancreatitis. Patients were excluded because of incomplete clinical evaluation (n = 13), tumor in the head of pancreas (n = 3), and post-pancreaticoduodenectomy (n = 1). In addition, patients with insufficient image quality (n = 12) were also excluded. Of 73 evaluable patients, 55 had single-episode acute pancreatitis (mean age, 57.5 years; range, 26-85 years) and 18 had recurrent acute pancreatitis (mean age, 46.1 years; range, 26-82 years). The single-episode acute pancreatitis subgroup included 16 female patients (mean age, 57.8 years; range, 16-85 years) and 39 men (mean age, 57.4 years; range, 24-82 years). The recurrent acute pancreatitis subgroup included 9 women (age, 26-82 years; mean, 50.7 years) and 9 men (age, 29-67 years; mean, 41.6 years). All of the patients with acute pancreatitis were Japanese (Tables 2 and 3).

Clinical findings

The accessory pancreatic duct was clearly visualized

Table 1 Characteristics of subjects in the community group with and without ansa pancreatica

	All subjects (n = 587)	Subjects without ansa pancreatica (n = 582)	Subjects with ansa pancreatica (n = 5)	P value	OR (95%CI)
Age (yr)	56.8 ± 10.4	56.8 ± 10.4	53.6 ± 10.7	0.60[1]	
Females	250 (43)	248 (43)	2 (40)	0.70[2]	
Brinkman index (cigarettes/d × years)	244 ± 408	246 ± 409	120 ± 240	0.36[1]	
Alcohol intake (kg/yr)	5.8 ± 7.8	5.7 ± 7.7	11.4 ± 12.9	0.43[1]	
Clinical history					
All cases of pancreatitis[3]	6 (1)	5 (0.9)	1 (20)	0.050[2]	28 (0.49-364)
Acute pancreatitis	4 (0.7)	3 (0.5)	1 (20)	0.034[2]	46 (0.74-746)
Diabetes mellitus	30 (5)	30 (5)	0	1[2]	
Hypertension	109 (19)	109 (19)	0	0.59[2]	
Hyperlipidemia	67 (11)	67 (12)	0	1[2]	
Any malignant neoplasm	45 (8)	45 (8)	0	1[2]	
Autoimmune disease	14 (2)	14 (2)	0	1[2]	

Values are presented as the n (%) or mean ± SD deviation. [1]Welch's t test; [2]Fisher's exact test; [3]Includes subjects with single-episode acute pancreatitis and subjects with recurrent acute pancreatitis.

Table 2 Characteristics of patients with single-episode acute pancreatitis with and without ansa pancreatica

	All patients (n = 55)	Patients without ansa pancreatica (n = 54)	Patients with ansa pancreatica (n = 1)
Age (yr)	57.5 ± 18.5	57.6 ± 18.5	50
Females	16 (29)	16 (30)	0 (0)
Brinkman index (cigarettes/d × years)	355 ± 887	364 ± 888	0
Alcohol intake (kg/yr)	8.1 ± 11.1	7.9 ± 11.1	16.1
Clinical history			
Diabetes mellitus	11 (20)	11 (22)	0 (0)
Hypertension	14 (25)	14 (29)	0 (0)
Hyperlipidemia	12 (22)	12 (25)	0 (0)
Any malignant neoplasm	8 (15)	8 (16)	0 (0)
Autoimmune disease	5 (9)	5 (10)	0 (0)

Values are presented as the n (%) or mean ± SD.

in 88.7% (587/663) of subjects in the community group and in 85.9% (73/85) of patients with acute pancreatitis, and was not significantly different between these two groups (P = 0.47; OR = 0.78; 95%CI: 0.40-1.67). In the community group, 0.85% (5/587) of subjects had ansa pancreatica; 17 (2.9%) had pancreas divisum, 37 (6.3%) had a meandering main pancreatic duct, 1 (0.017%) had an anomalous arrangement of the pancreaticobiliary ductal system, and 1 (0.017%) had a retroportal main pancreatic duct.

When we compared subjects with and without ansa pancreatica in the community group, we observed no significant differences between these two subgroups in terms of sex, clinical history, or hematologic and biochemical variables (Table 1). The incidence of acute pancreatitis, including single-episode acute pancreatitis and recurrent acute pancreatitis, was greater in subjects with ansa pancreatica (20.0%, 1/5) than in subjects without ansa pancreatica (0.52%, 3/582), although this difference did not reach statistical significance (Table 1). Two of the subjects (40%, 2/5) with ansa pancreatica in the community group presented with other radiological abnormalities in the pancreatic duct: one had slight dilation of main pancreatic duct (4 mm) and the other had Wirsungocele.

Among the patients with acute pancreatitis, 3 (4.1%, 3/73) had ansa pancreatica, of which 2 (11.1%, 2/18) had recurrent acute pancreatitis and 1 (1.8%, 1/55) had single-episode acute pancreatitis. Pancreatitis in patients with ansa pancreatica was caused by alcohol in two patients and was idiopathic in one (Table 4). None of the patients with ansa pancreatica in the acute pancreatitis group had other accompanying morphological pancreaticobiliary abnormalities.

Compared with the community group, the recurrent acute pancreatitis subgroup showed significantly higher rates of ansa pancreatica after family-wise correction, with a very high OR (P = 0.016; OR = 14.3; 95%CI: 1.27-96.1). However, no difference was observed in the single-episode acute pancreatitis subgroup or the total group of patients with acute pancreatitis (Table 5). The age and alcohol intake were significantly lower and the frequency of autoimmune disease (including non-organ-specific autoimmune disorders and organ-specific autoimmune disorders like autoimmune pancreatitis) was significantly greater in the recurrent acute pancreatitis subgroup than in the community group, but no differences were observed in the other clinical features (Table 6). Based on the results of the univariate analyses, multiple logistic regression analyses were performed using age, alcohol intake, presence of ansa pancreatica, and presence of autoimmune disease as independent covariates. Considering that the exact type of pancreatitis (recurrent or single-episode) was unknown in four subjects with acute pancreatitis in the community group, we performed statistical analyses using all combinations of recurrent or single-episode acute

Table 3 Characteristics of subjects with recurrent acute pancreatitis with and without ansa pancreatica

	All patients (n = 18)	Patients without ansa pancreatica (n = 16)	Patients with ansa pancreatica (n = 2)	P value
Age (yr)	46.1 ± 14.4	44.6 ± 14.1	58.5 ± 9.5	0.36[1]
Female	9 (50)	8 (50)	1 (50)	1[2]
Brinkman index (cigarettes/d × years)	103.1 ± 174	122 ± 183	0 ± 0	0.06[1]
Alcohol intake (kg/yr)	1.06 ± 2.01	1.14 ± 2.1	0	0.62[1]
Clinical history				
Diabetes mellitus	1 (6)	0 (0)	1 (50)	0.19[2]
Hypertension	3 (17)	3 (19)	0 (0)	1[1,2]
Hyperlipidemia	5 (28)	5 (31)	0 (0)	1[1]
Any malignant neoplasm	0 (0)	0 (0)	0 (0)	1[1]
Autoimmune disease	4 (22)	4 (25)	0 (0)	1[1]

Values are presented as the n (%) or mean ± SD. [1]Welch's t test; [2]Fisher's exact test.

Table 4 Causes of pancreatitis and ansa pancreatica

Cause	All patients with acute pancreatitis (n = 73)	Patients with single-episode acute pancreatitis (n = 55)	Patients with recurrent acute pancreatitis (n = 18)
Gallstones	19 (26.0)	18 (33.0)	1 (5.6)
Alcohol	14 (19.0)[2]	12 (22.0)[1]	2 (11.0)[1]
Idiopathic	10 (14.0)[1]	6 (11.0)	4 (22.0)[1]
Iatrogenic	6 (8.2)	6 (11.0)	0 (0)
Pancreas divisum[1]	6 (8.2)	1 (1.8)	5 (28.0)
Autoimmunity[2]	4 (5.6)	2 (3.6)	2 (5.6)
Meandering main pancreatic duct[3]	3 (4.2)	1 (1.8)	2 (11.0)
Pancreaticobiliary maljunction	1 (1.4)	1 (1.8)	0 (0)
Alcohol and hyperlipidemia combined	1 (1.4)	1 (1.8)	0 (0)
Choledocal cyst, pancreaticobiliary maljunction, and pancreas divisum combined	1 (1.4)	1 (1.8)	0 (0)
Cholesterol embolism	1 (1.4)	1 (1.8)	0 (0)
Crohn's disease	1 (1.4)	1 (1.8)	0 (0)
Drug induced	1 (1.4)	1 (1.8)	0 (0)
Hyperlipidemia	3 (4.2)	2 (3.6)	1 (5.6)
Hypothermia	1 (1.4)	1 (1.8)	0 (0)
Sphincter of Oddi dysfunction	1 (1.4)	0 (0)	1 (5.6)

Values are presented as the n (%); values in square brackets represent the number of subjects with ansa pancreatica. [1]Diagnosed according to the criteria used in a previous study[8]; [2]Diagnosed according to the Asian Criteria of Autoimmune Pancreatitis revised in 2008[27]; [3]Diagnosed according to the criteria used in a previous study[9].

Table 5 Frequency of ansa pancreatica and its association with single-episode and recurrent acute pancreatitis

	Cases of ansa pancreatica (%)	P value[1]	OR (95%CI)
All patients with acute pancreatitis	3/73 (4.1)	0.048	4.97 (0.76-26.2)
Patients with single-episode acute pancreatitis	1/55 (1.8)	0.42	2.12 (0.04-19.7)
Patients with recurrent acute pancreatitis	2/18 (11.1)	0.016[2]	14.3 (1.27-96.1)
Community group	5/587 (0.85)		

[1]Fisher's exact test for comparisons with the community group; [2]Statistically significant after family-wise correction.

pancreatitis. These analyses revealed a significant positive association between ansa pancreatica and the onset of recurrent acute pancreatitis in all combinations, with ORs ranging from 14.0 (P = 0.03; 95%CI: 3.0-25.0) to 79.3 (P = 0.0002; 95%CI: 69.5-89.1) depending on the combination tested. The presence of autoimmune disease was also significantly associated with the onset of recurrent acute pancreatitis in all combinations, with ORs ranging from 13.2 (P = 0.0030; 995%CI: 7.7-18.7) to 18.4 (P = 0.0028; 95%CI: 11.6-25.2). According to the results of multiple logistic regression analyses, neither age nor alcohol intake were significantly associated with the onset of recurrent acute pancreatitis.

When we evaluated the prevalence of ansa pancreatica according to the cause of pancreatitis, we found that ansa pancreatica was most frequent in patients with alcoholic pancreatitis (14.3%, 2/14) (Table 4).

Among all patients with acute pancreatitis, MRCP scans were obtained in the acute phase of pancreatitis

Table 6 Comparison of the characteristics of patients with recurrent acute pancreatitis and the community group

	Community group (n = 587)	Patients with recurrent acute pancreatitis (n = 18)	P value	OR (95%CI)
Age (yr)	56.8 ± 10.4	46.1 ± 14.4	0.0001[1,2]	
Female	250 (43)	9 (50)	0.63[3]	
Brinkman index (cigarettes/d × years)	244 ± 408	103.1 ± 174	0.019[1]	
Alcohol intake (kg/yr)	5.8 ± 7.8	1.06 ± 2.0	< 0.0001[1,2]	
Clinical history				
Diabetes mellitus	30 (5)	1 (6)	1[3]	
Hypertension	109 (19)	3 (17)	1[3]	
Hyperlipidemia	67 (11)	5 (28)	0.051[3]	
Any malignant neoplasm	45 (8)	0 (0)	0.39[3]	
Autoimmune disease	14 (2)	4 (22)	0.0010[2,3]	11.6 (2.46-43.7)

Values are presented as the n (%) or mean ± SD. [1]Welch's t test; [2]Statistically significant after family-wise correction; [3]Fisher's exact test.

in 1 (33.3%) patient with ansa pancreatica. This patient had an idiopathic recurrent acute attack, which presented with pancreatitis limited to the head of pancreas and was classified as non-severe[19].

In all eight cases with ansa pancreatica, the duct arose from the papillary side of the flexion point of the ventral duct where the normal accessory duct arose (Figures 1 and 2).

DISCUSSION

To our knowledge, this is the first case-control study to focus on ansa pancreatica. We determined the prevalence of ansa pancreatica visible on MRCP in a community group and in patients with single-episode or recurrent acute pancreatitis, and revealed a significant association between the presence of ansa pancreatica and the onset of recurrent acute pancreatitis.

MRCP is a diagnostic technique that can image the pancreaticobiliary duct in a non-invasive manner; it does not require radiation exposure or injection of contrast media, and carries a low risk of complications. In recent years, technological advances mean that MRCP has started to compete with ERCP in terms of imaging quality[20,21]. In the present study, we used MRCP, which enabled us to acquire pancreaticobiliary images of the study groups in nearly identical conditions, which is necessary in a case-control study.

Ansa pancreatica was characterized by the absent accessory duct at the junction with the ventral duct and by the presence of an extra curved duct linking the ventral and dorsal pancreatic duct. In this study, we observed that all ansa pancreatica ducts arose from the papillary side of the flexion point of the ventral duct. This location was similar to the bifurcation of the lower branch of ventral duct, which arose from a point closer to the papillary side than the source of the normal accessory duct. Dawson proposed that ansa pancreatica was formed by the fusion of the proximal part of the dorsal duct with the lower branches of the dorsal and ventral ducts[12], and this hypothesis was consistent with our findings.

In several case reports, it was speculated that the presence of ansa pancreatica is a predisposing factor for pancreatitis. Kamisawa and Dawson reported a more frequent occurrence of impervious minor papilla in patients with ansa pancreatica (66.7%-79.3%[12,22]) than in healthy subjects (59%)[13], and this was assumed to be the cause of pancreatitis. In the present study, patients with recurrent acute pancreatitis had a significantly higher frequency of ansa pancreatica (11.1%) than seen in the community group (0.85%). This result indicates that the presence of ansa pancreatica is a predisposing factor for pancreatitis, as previously hypothesized.

Dawson and Kamisawa reported that in subjects without pancreatitis, the ansa pancreatica type of accessory duct was detected in 17% and 13.6% by ERCP and eosine injection, respectively[12,22]; these values are considerably higher than the values in our study. We found that most of the ansa pancreatica ducts presented as faint outlines on the MRCP images. Therefore, the detectability of ansa pancreatica may be influenced by differences in imaging methods, because intraductal pressure is higher during ERCP than in normal physiological conditions[23]. We also speculate that racial difference may have influenced the results. In addition, we think that the ansa pancreatica detected on MRCP are more dilated cases reflecting more severe congestion of pancreatic juice and high intraductal pressure, which are relevant to the etiology of pancreatitis.

Ansa pancreatica is widely assumed to be a predisposing factor for pancreatitis, particularly in heavy alcohol consumers[24-26]. Indeed, we found that the frequency of ansa pancreatica was highest in patients with alcoholic pancreatitis, although this was not statistically significant. This result may indicate that heavy alcohol consumers with ansa pancreatica should be advised to abstain from drinking. Nevertheless, the exact mechanism linking alcohol consumption and ansa pancreatica in the onset of pancreatitis is unknown.

We also observed that patients with recurrent acute pancreatitis were significantly more likely to have an autoimmune disease than subjects in the community group. This seems reasonable because autoimmune pancreatitis shows a strong tendency to recur.

The major limitations of the present study are as follows. First, the ability of MRCP to assess ductal anatomy might be imperfect. However, MRCP is the only pancreaticobiliary imaging method that can be used in healthy subjects and was therefore essential to ensure the procedures were comparable in this case-control study. Second, the subjects enrolled in this study were Asian: almost all subjects were Japanese. Therefore, careful consideration is needed in generalizing this result to the whole population.

In conclusion, this is the first study to investigate the clinical significance of ansa pancreatica using a case-control study design. Our results indicate that the presence of ansa pancreatica is a predisposing factor for the onset of recurrent acute pancreatitis.

COMMENTS

Background

Ansa pancreatica is a rare anatomical variation of the accessory pancreatic duct. Its *in vivo* prevalence and clinical significance were unknown.

Research frontiers

Ansa pancreatica was hypothesized to be a predisposing factor for pancreatitis, however no case-control study has confirmed it. This study contributes to reveal the association of the onset of recurrent acute pancreatitis and the presence of ansa pancreatica.

Innovations and breakthrough

This study is the first case-control study to determine the non-biased prevalence and provide evidence that the presence of ansa pancreatica is significantly associated with recurrent acute pancreatitis, using non-invasive magnetic resonance imaging.

Applications

This study investigated ansa pancreatica was a predisposing factor for recurrent acute pancreatitis. If a patient with ansa pancreatica has acute pancreatitis, the risk of recurrence should be noticed.

Peer-review

Ansa pancreatica is in fact said to be a rare anatomical variation of pancreatic ducts. This paper is the first in the world study of this subject, where it was indicated that ansa pancreatica is to be predisposing factor for recurrent acute pancreatitis.

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P- Reviewer: Jagielski M, Liu B **S- Editor**: Gong ZM
L- Editor: A **E- Editor**: Zhang FF

World Journal of Gastroenterology

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Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.3748/wjg.v22.i40.8949

World J Gastroenterol 2016 October 28; 22(40): 8949-8955
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

ORIGINAL ARTICLE

Retrospective Study

Fibrosis in nonalcoholic fatty liver disease: Noninvasive assessment using computed tomography volumetry

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Supported by a Grant-in-in-Aid for Scientific Research (C) (No. 26461796) from the Japanese Ministry of Education, Culture, Sports, Science, and Technology.

Institutional review board statement: This study was reviewed and approved by the Ethics Committee of the Kyushu University.

Informed consent statement: The requirements for informed consent were waived due to the retrospective design. The details of the study are published on the home page of Kyushu University.

Conflict-of-interest statement: One author (Fujioka T) is an employee of Philips Electronics Japan.

Data sharing statement: No additional data are available.

Manuscript source: Invited manuscript

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Received: June 24, 2016
Peer-review started: June 28, 2016
First decision: August 22, 2016
Revised: September 2, 2016
Accepted: September 28, 2016

Article in press: September 28, 2016
Published online: October 28, 2016

Abstract

AIM

To evaluate the diagnostic performance of computed tomography (CT) volumetry for discriminating the fibrosis stage in patients with nonalcoholic fatty liver disease (NAFLD).

METHODS

A total of 38 NAFLD patients were enrolled. On the basis of CT imaging, the volumes of total, left lateral segment (LLS), left medial segment, caudate lobe, and right lobe (RL) of the liver were calculated with a dedicated liver application. The relationship between the volume percentage of each area and fibrosis stage was analyzed using Spearman's rank correlation coefficient. A receiver operating characteristic (ROC) curve analysis was performed to determine the accuracy of CT volumetry for discriminating fibrosis stage.

RESULTS

The volume percentages of the caudate lobe and the LLS significantly increased with the fibrosis stage (r = 0.815, P < 0.001; and r = 0.465, P = 0.003, respectively). Contrarily, the volume percentage of the RL significantly decreased with fibrosis stage (r = -0.563, P < 0.001). The volume percentage of the caudate lobe had the best diagnostic accuracy for staging fibrosis, and the area under the ROC curve values for discriminating fibrosis stage were as follows: ≥ F1, 0.896; ≥ F2, 0.929; ≥ F3, 0.955; and ≥ F4, 0.923. The best cut-off for advanced fibrosis (F3-F4) was 4.789%, 85.7% sensitivity and 94.1% specificity.

CONCLUSION

The volume percentage of the caudate lobe calculated by CT volumetry is a useful diagnostic parameter for staging fibrosis in NAFLD patients.

Key words: Nonalcoholic fatty liver disease; Computed tomography volumetry; Fibrosis stage; Nonalcoholic steatohepatitis

Core tip: This is a retrospective study to elucidate the morphological change in nonalcoholic fatty liver disease (NAFLD) using computed tomography (CT) volumetry and to evaluate the diagnostic performance of CT volumetry for discriminating the fibrosis stage in NAFLD. The volume percentages of the caudate lobe calculated by CT volumetry were significantly increased with the increase in fibrosis stage in NAFLD. The volume percentage of the caudate lobe is a useful diagnostic parameter for staging fibrosis in patients with NAFLD. The evaluation of liver volume using CT volumetry is useful for predicting the fibrosis stage in NAFLD.

Fujita N, Nishie A, Asayama Y, Ishigami K, Ushijima Y, Takayama Y, Okamoto D, Shirabe K, Yoshizumi T, Kotoh K, Furusyo N, Hida T, Oda Y, Fujioka T, Honda H. Fibrosis in nonalcoholic fatty liver disease: Noninvasive assessment using computed tomography volumetry. *World J Gastroenterol* 2016; 22(40): 8949-8955 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i40/8949.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i40.8949

INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is currently the most prevalent liver disease worldwide with a prevalence of 20%-30%[1,2]. The spectrum of NAFLD ranges from simple steatosis to nonalcoholic steatohepatitis (NASH), which can progress to end-stage cirrhosis[1,3]. It has been reported that advanced fibrosis or cirrhosis of NAFLD represents a clear worsening of prognosis[4]. Monitoring the fibrosis stage is therefore important in the treatment of NAFLD.

A liver biopsy is considered the reference standard for the diagnosis of NAFLD, but repeating a liver biopsy is not desirable because of the risk of complications and the costs[5]. A noninvasive tool for the diagnosis of NAFLD would be clinically useful, and the utility of magnetic resonance (MR) imaging for detecting the fibrosis stage in NAFLD has been reported[6-8]. To the best of our knowledge, there have been no studies describing the diagnostic feasibility of computed tomography (CT) imaging for assessment of the fibrosis stage of NAFLD.

Generally, the morphology of the liver changes as the fibrosis stage advances, and its pattern depends on the primary disease. The morphological change of the liver as the fibrosis stage advances in other liver diseases such as chronic viral hepatitis, alcoholic hepatitis, primary biliary cirrhosis, primary sclerosing cholangitis and autoimmune hepatitis has been well documented[9], but the morphologic change of the liver caused by NAFLD is still unclear. Multidetector CT (MDCT) has been demonstrated to measure liver volume correctly, and CT volumetry has been used widely- especially as a method for preoperatively assessing the volume of the liver[10]. It would be clinically useful to be able to use CT volumetry to elucidate this morphological change and to predict the fibrosis stage in NAFLD noninvasively.

Our purposes in the present study were thus to elucidate the morphological change in NAFLD using CT volumetry and to evaluate the diagnostic performance of CT volumetry for discriminating the fibrosis stage in

Table 1 Computed tomography parameters

CT scanner	4-slice MDCT			64- or 256- or 320- slice MDCT				
No. of patients	4	2	5	5	2	10	4	6
Contrast material (mgI/mL)	370	300	370	370	370	370	300	370
Contrast material dose	100 mL	100 mL	600 mgI/kg	100 mL	100 mL	600 mgI/kg	100 mL	600 mgI/kg
Injection rate	2.5 mL/s	2.5 mL/s	20 s[1]	3 mL/s	2.5 mL/s	30 s[1]	2.5 mL/s	20 s[1]
Portal phase delay (s)	70	70	55	70	70	60	60	70
Tube voltage (kVp)	120	120	120	120	120	120	120	120
Tube current (mAs)	300	300	300	Auto	Auto	Auto	Auto	Auto
Reconstruction thickenss (mm)	5	3	3	5	3	1	5	2

[1]Injection at a variable rate with a fixed duration. MDCT: Multidetector CT; CT: Computed tomography.

NAFLD.

MATERIALS AND METHODS

Patients

Our institutional review board approved this study, and the requirements for informed consent were waived due to the retrospective design.

We enrolled 38 patients who underwent contrast-enhanced CT and were diagnosed as having NAFLD based on histological findings from March 2004 to August 2013 at our institution. All patients had no alcohol consumption habit and no evidence of a specific cause for liver disease such as viral hepatitis B or C, hemochromatosis, or autoimmune or cholestatic liver disease. Of the 38 patients, 20 were men and 18 were women. The mean age was 50.7 years, ranging from 20 to 82 years. The mean body mass index was 26.6 kg/m^2, ranging from 20.3 to 36.8 kg/m^2. The prevalence of diabetes mellitus was 36.8% (14 patients); that of dyslipidemia was 18.4% (7 patients), and that of hypertension was 31.6% (12 patients). Twenty-eight patients were diagnosed as having NAFLD based on liver biopsy, and the diagnoses of the other 10 patients were based on a surgically resected specimen. Of the 10 patients who underwent surgery, liver resection was performed in three patients for hepatocellular carcinoma (HCC), and liver transplantation was performed in seven patients for decompensated cirrhosis.

CT protocol

Because we used a retrospective design, various types of CT scanners and different CT protocols had been used. Dynamic CT studies were performed with a 4-slice (Aquilion: Toshiba Medical Systems, Tokyo: n = 9; or Somatom Plus 4 Volume Zoom: Siemens-Asahi Medical Technologies, Tokyo: n = 2), 64-slice (Aquilion: Toshiba Medical Systems: n = 16; or Brilliance 64: Philips, Cleveland OH, United States: n = 5), 256-slice (Brilliance iCT: Philips: n = 4) or 320-slice (Aquilion One: Toshiba Medical Systems: n = 2) MDCT scanner. Each patient received intravenous nonionic contrast material containing 300 mgI/mL or 370 mgI/mL iopamidol (Iopamiron; Bayer, Osaka, Japan) by an automated power injector, and the portal phase was acquired. The details of the CT protocols are shown in Table 1. The interval between the preoperative MDCT study and the biopsy or surgery ranged from 1 to 244 d (mean ± SD, 44.0 ± 56.7 d).

Image analysis

Two radiologists (Nishie A and Fujita N, with 21 and 12 years of experience in abdominal imaging, respectively) who were blinded to the clinical and pathologic results measured the liver segment volumes on portal phase images in a consensus fashion. The portal phase data were transferred to a workstation (Intellispace Portal 6.0, Philips) and analyzed with a dedicated liver application (Liver Analysis: Philips).

First, the total liver and vessels (hepatic and portal venous trees) were segmented automatically. If liver lesions such as a hepatic cyst, cavernous hemangioma or HCC were present, the lesions were segmented semi-automatically and subtracted from the liver volume. In this study, we subtracted the volume of live lesions with a diameter < 5 cm.

Second, we calculated the volumes of the total, the left lateral segment (LLS), the left medial segment (LMS), the caudate lobe, and the right lobe (RL) of the liver semi-automatically using the Philips "Liver Analysis" application by the position of 10 anatomical landmarks: inferior vena cava, right portal bifurcation, right hepatic vein, middle hepatic vein, umbilical fissure, left portal bifurcation, tip left liver, superficial ligamentum venosum, deep ligamentum venosum, and superior deep ligamentum venosum. If corrections were necessary, we corrected the data manually on the application to achieve a precise final result. We then determined the volume percentage of the LLS (LLS volume/total volume), LMS (LMS volume/total volume), caudate lobe (caudate lobe volume/total volume) and RL (RL volume/total volume).

Histopathologic analysis

All of the liver specimens were reviewed by one pathologist (Hida T) who was blinded to the patients' information. The criteria for the fibrosis severity of

Table 2 Correlation between liver volume and fibrosis stage

Parameter	F0 (n = 11)	F1 (n = 5)	F2 (n = 1)	F3 (n = 9)	F4 (n = 12)	r	P value
Total volume (cm^3)	1252.3 ± 155.1	1280.4 ± 240.0	1235.8	1364.5 ± 320.0	1030.2 ± 366.1	-0.193	NS
LLS (%)	18.3 ± 1.9	22.1 ± 3.9	21.5	25.3 ± 8.8	26.5 ± 8.8	0.465	0.003
LMS (%)	12.6 ± 1.7	12.0 ± 3.7	16.2	11.3 ± 2.8	11.1 ± 3.5	-0.248	NS
Caudate lobe (%)	3.0 ± 1.2	3.7 ± 0.8	3.2	5.3 ± 1.0	9.3 ± 4.1	0.815	< 0.001
RL (%)	66.1 ± 2.2	62.1 ± 5.6	59.1	58.0 ± 10.4	52.7 ± 10.5	-0.563	< 0.001

Continuous data are mean ± SD. LLS: Left lateral segment; LMS: Left medial segment; RL: Right lobe.

Table 3 Receiver operating characteristic analysis of the diagnostic performance of volume percentage of caudate lobe for hepatic fibrosis

	F0 *vs* ⩾ F1	⩽ F1 *vs* ⩾ F2	⩽ F2 *vs* ⩾ F3	⩽ F3 *vs* F4
Az value	0.896	0.929	0.955	0.923
Cutoff value	3.957	4.789	4.789	5.834
Sensitivity (%)	88.9	81.8	85.7	83.3
Specificity (%)	90.9	93.8	94.1	88.4
Accuracy (%)	89.4	86.8	89.4	86.8
PPV (%)	96.0	94.7	94.7	76.9

PPV: Positive predictive value; NPV: Negative predictive value.

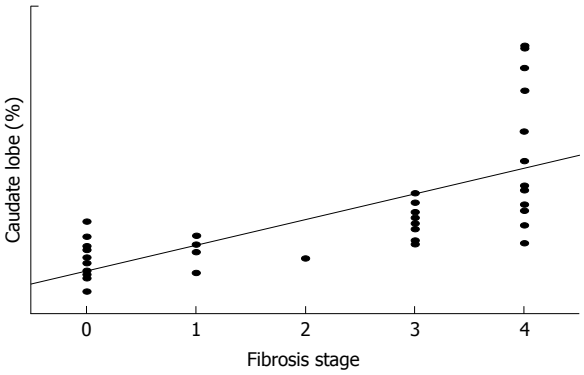

Figure 1 Correlation between volume percentage of caudate lobe and fibrosis stage. Strong correlation was observed between liver fibrosis stage and volume percentage of caudate lobe (P < 0.001, r = 0.815).

NAFLD was based on the classification by Brunt *et al*[11], and liver fibrosis was staged as follows: stage 0 (F0), no fibrosis; stage 1 (F1), zone 3 perisinusoidal/pericellular fibrosis; stage 2 (F2), zone 3 perisinusoidal/pericellular fibrosis with focal or extensive periportal fibrosis; stage 3 (F3), zone 3 perisinusoidal/pericellular fibrosis and portal fibrosis with focal or extensive bridging fibrosis; stage 4 (F4), liver cirrhosis[11].

Statistical analyses

We analyzed the correlation between the volume percentage of each region and the fibrosis stage by Spearman's rank correlation test. A correlation was considered strong if the absolute value of the correlation coefficient (r) was > 0.7, moderate if the r was 0.4-0.7, weak if the r was 0.2-0.4, and absent if the r was < 0.2. The diagnostic accuracy of volume percentage was assessed by a receiver operating characteristic curve analysis. We calculated the area under the curve (Az value) and the optimal cutoff value for differentiating ⩾ F1 from F0, ⩾ F2 from ⩽ F1, ⩾ F3 from ⩽ F2, and F4 from ⩽ F3. Standard definitions were used for the calculation of the sensitivity, specificity, accuracy, positive predictive value and negative predictive value. JMP 11.0.0 software (SAS Institute, Cary, NC, United States) was used for the analyses. P values < 0.05 were considered significant.

RESULTS

Histological findings

Of the 38 patients with histological data, the distribution of fibrosis stage was as follows: F0 in 28.9% (11/38), F1 in 13.2% (5/38), F2 in 2.6% (1/38), F3 in 23.7% (9/38), and F4 in 31.6% (12/38).

The liver volume percentage and the fibrosis stage

Table 2 provides the correlations between liver volumes and fibrosis stage. With the increase in the liver fibrosis stage, the volume percentages of the LLS and the caudate lobe increased significantly (P = 0.003, r = 0.465 and P < 0.001, r = 0.815, respectively) and that of the RL decreased significantly (P < 0.001, r = -0.563). A strong correlation was observed between the liver fibrosis stage and the volume percentage of the caudate lobe (Figure 1). There was no correlation between fibrosis stage and the total volume or the volume percentage of the LMS.

Table 3 summarizes the diagnostic performance of the volume percentage of the caudate lobe for predicting the fibrosis stage. The volume percentage of the caudate lobe was the best to discriminate ⩾ F3 from ⩽ F2, with an Az value of 0.955.

Figures 2-4 present representative patient images.

DISCUSSION

The major causes of liver fibrosis include hepatitis B and C, alcohol abuse, primary sclerosing cholangitis, primary biliary cirrhosis and autoimmune hepatitis. In such diseases, the morphologic change of the liver with the advance in fibrosis stage has been well reported[9]. However, to the best of our knowledge, the morphologic change of the liver with the advanced

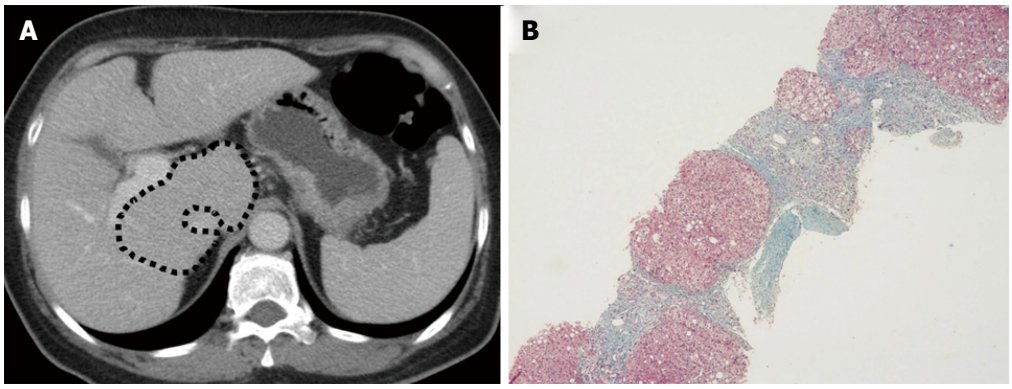

Figure 2 A 60-year-old woman with nonalcoholic steatohepatitis, fibrosis stage 4. The total volume of the liver was 1169.4 mL, and the volume percentages of the left lateral segment, left medial segment, caudate lobe and right lobe were 26.6%, 18.7%, 12.9% and 41.8%, respectively (A). The dotted line shows the caudate lobe. The biopsy specimen showed liver cirrhosis with regenerative nodules (B).

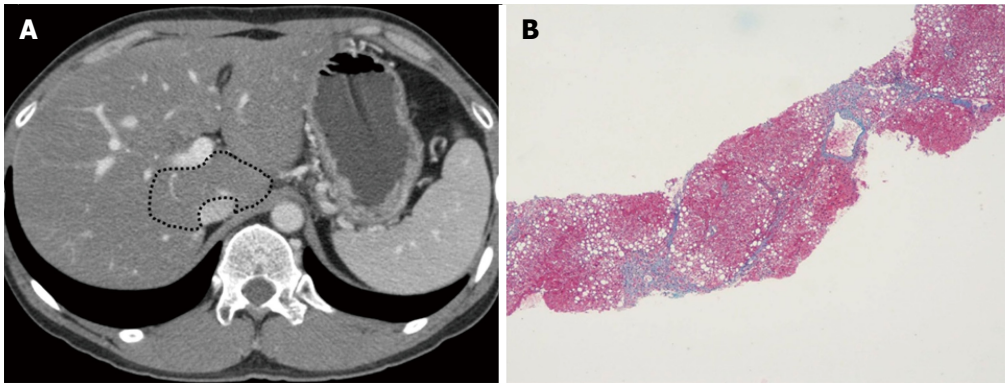

Figure 3 A 39-year-old man with nonalcoholic steatohepatitis, fibrosis stage 3. The total volume of the liver was 1805.4 mL, and the volume percentages of the left lateral segment, left medial segment, caudate lobe and right lobe were 21.3%, 12.3%, 5.6% and 60.8% respectively (A). The dotted line shows the caudate lobe. The biopsy specimen showed bridging fibrosis (B).

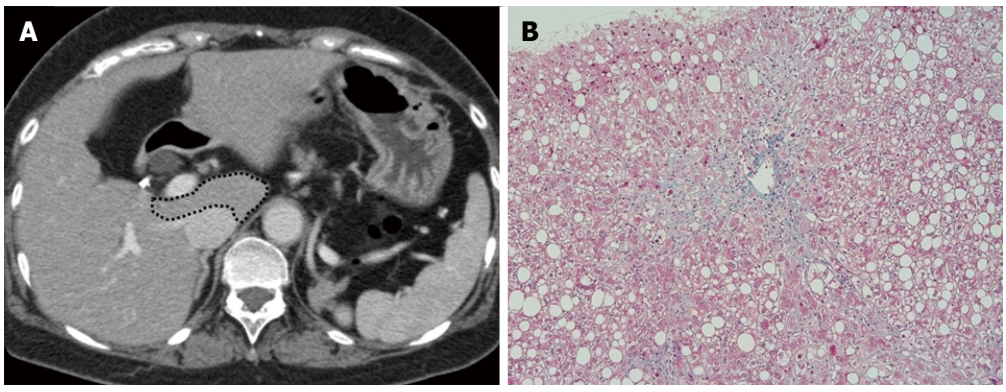

Figure 4 A 63-year-old woman with nonalcoholic steatohepatitis, fibrosis stage 1. The total volume of the liver was 1190.9 mL, and volume percentages of the left lateral segment, left medial segment, caudate lobe and right lobe were 21.8%, 13.7%, 4.3% and 60.2%, respectively (A). The dotted line shows the caudate lobe. The biopsy specimen showed pericellular fibrosis at zone 3 (B).

stage of NAFLD is still unclear. In the present study, as the fibrosis stage advanced, the volume percentage of the LLS and the caudate lobe increased significantly, and that of the RL decreased significantly in the NAFLD patients.

Focal hypertrophy of the caudate lobe or LLS and atrophy of the RL are common findings in liver cirrhosis[9]. However, a strong correlation was observed between fibrosis stage and the volume percentage of the caudate lobe in our study. Ozaki *et al*[12] recently reported that hypertrophy of the caudate lobe progressed more in alcoholism and NASH patients than in virus-related etiologies in patients with liver cirrhosis, Child-Pugh Class A. It was also reported

that enlargement of the caudate lobe was a more frequent finding in alcoholic cirrhosis than virus-induced cirrhosis[13]. Considering our present findings and the above reports, we suggest that hypertrophy of the caudate lobe is a characteristic change of NAFLD as well as alcoholism, despite their distinctly different clinical histories.

It has been reported that fibrosis in NASH worsened in 30%-40% and 5%-25% of NASH cases that advanced to liver cirrhosis over a period of 5-10 years[14,15]. Advanced fibrosis (≥ F3) or cirrhosis (F4) of NASH represents a clear worsening of prognosis[4]. Liver biopsy has been considered the reference standard in the assessment of liver fibrosis in NALFD, but it is invasive and cannot be repeated frequently. Several noninvasive imaging methods have thus been developed to estimate liver fibrosis in NAFLD. Kim *et al*[6] reported that MR elastography was a useful diagnostic tool for detecting advanced fibrosis in NAFLD (≥ F3, Az value = 0.954). Ding *et al*[7] reported the usefulness of T1 mapping on Gd-EOB-DTPA-enhanced MR imaging. They asserted that both the T1 relaxation times of the liver parenchyma and the decreased rate were useful to diagnose advanced fibrosis in NAFLD (≥ F3, Az value = 0.95 and Az value = 0.95, respectively).

However, these imaging techniques are not widely spread and may not be suitable for routine imaging examinations. The results of our present study indicate that the volume percentage of the caudate lobe has a high diagnostic performance for the fibrous staging of NAFLD (Az value = 0.955). The Az value that we obtained in this study is equivalent to that of previous studies using MR imaging[6,7]. This suggests that routine CT imaging using a combination of CT volumetry would be an effective and noninvasive way to diagnose hepatic fibrosis in NAFLD.

CT volumetry is now widely used for the pre-operative volumetric assessment of the liver[10]. Traditionally, CT volumetry is performed by manually tracing the liver and by the summation of the liver volume in axial sections. However, such manual methods are operator-dependent and require a significant amount of time and attention. Automated and semi-automated versions of CT volumetry have been proposed, but automatic CT volumetry may tend to fail for CT images that are low-contrast and have missing edges due to similar intensities of adjacent organs. In the present study, we used a semi-automated CT volumetry method that provides more flexibility than an automated method. Indeed, Gotra *et al*[16] reported that a semi-automated method substantially shortened the interaction time while preserving high repeatability and agreement with manual volumetry.

Our study has some limitations. First, our population of 38 patients was small, and the number of cases with each degree of fibrosis was not uniform. Second, we diagnosed the fibrosis stage of NALFD based on a liver biopsy in 28 of the 38 patients. Assessments of liver biopsy results can have high inter- and intraobserver variability[17], and the reproducibility of the histological fibrosis staging could not be examined in this study. Third, we used a retrospective design, and various types of CT scanners and different CT protocols were used.

In conclusion, the volume percentages of the LLS and the caudate lobe calculated by CT volumetry were significantly increased and that of the RL was significantly decreased with the increase in fibrosis stage in NAFLD. The volume percentage of the caudate lobe is a useful diagnostic parameter for staging fibrosis in patients with NAFLD. The evaluation of liver volume using CT volumetry is useful for predicting the fibrosis stage in NAFLD.

ACKNOWLEDGMENTS

We thank Dr. Yoshihiko Maehara, Department of Surgery and Science, Kyushu University, for providing the clinical information for this manuscript.

COMMENTS

Background

Nonalcoholic fatty liver disease (NAFLD) is currently the most prevalent liver disease worldwide. Advanced fibrosis or cirrhosis of NAFLD represents a clear worsening of prognosis. Monitoring the fibrosis stage is important in the treatment of NAFLD. The morphological change of the liver as the fibrosis stage advances in NAFLD is still unclear. In this study, we elucidated the morphological change in NAFLD using computed tomography (CT) volumetry and evaluated the diagnostic performance of CT volumetry for discriminating the fibrosis stage in NAFLD.

Research frontiers

CT volumetry has been used as a method for assessing the volume of the liver. The results of this study contribute to clarifying the diagnostic potential of CT volumetry for the fibrosis stage in NAFLD.

Innovations and breakthroughs

These results indicate that the volume percentage of the caudate lobe has a high diagnostic performance for the fibrous staging of NAFLD (≥ F3 from ≤ F2, Az value = 0.955).

Applications

This study suggests that the evaluation of liver volume using CT volumetry is useful for predicting the fibrosis stage in NAFLD.

Terminology

CT volumetry: A method that enables assessment the volume of the liver.

Peer-review

Recommend of the manuscript is to be accepted, despite several limitations of the study that you already mentioned on the DISCUSSION section.

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DOI: 10.3748/wjg.v22.i40.8956

World J Gastroenterol 2016 October 28; 22(40): 8956-8966
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

ORIGINAL ARTICLE

Retrospective Study

Neuroendocrine neoplasms of liver - A 5-year retrospective clinico-pathological study applying World Health Organization 2010 classification

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Author contributions: Burad DK contributed to data collection, data analysis and wrote the paper; Kodiatte TA contributed to data analysis and references; Rajeeb SM contributed to clinical data collection and analysis; Goel A analysed the clinical data and wrote the clinical part; Eapen CE provided clinical advice; Ramakrishna B designed and supervised the study, and wrote the paper.

Institutional review board statement: This study was approved by Institutional review board of Christian Medical College, Vellore, India.

Informed consent statement: This study is exempt from informed consent, since it is a retrospective study and the data collection and analysis were carried out without disclosing patient's identity.

Conflict-of-interest statement: All the authors declare that there are no commercial, personal and any other potential conflicting interests related to the submitted manuscript.

Data sharing statement: No additional data are available.

Manuscript source: Invited manuscript

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Received: June 15, 2016
Peer-review started: June 17, 2016
First decision: July 12, 2016
Revised: July 29, 2016
Accepted: August 23, 2016
Article in press: August 23, 2016
Published online: October 28, 2016

Abstract

AIM

To study the clinicopathological characteristics of neuroendocrine neoplasms (NEN) on liver samples and apply World Health Organization (WHO) 2010 grading of gastroenteropancreatic (GEP) NEN.

METHODS

Clinicopathological features of 79 cases of NEN of the liver diagnosed between January 2011 to December 2015 were analyzed. WHO 2010 classification of GEP NEN was applied and the tumors were graded as G1, G2 or G3. Two more categories, D1/2 (discordant 1/2) and D2/3 (discordant 2/3) were also applied. The D1/2 grade tumors had a mitotic count of G1 and Ki-67 index of G2. The D2/3 tumors had a mitotic count of G2 and Ki-67 index of G3. The follow up details which were available till the end of the study period (December 2015) were collected.

RESULTS

Of the 79 tumors, 16 each were G1 and G2, and 18 were G3 tumors. Of the remaining 29 tumors, 13 were assigned to D1/2 and 16 were D2/3 grade. Male preponderance was noted in all tumors except for G2 neoplasms, which showed a slight female predilection. The median age at presentation was 47 years (range 10-82 years). The most common presentation was abdominal pain (81%). Pancreas (49%) was the most common site of primary followed by gastrointestinal tract (24.4%) and lungs (18%). Radiologically, 87% of the patients had multiple liver lesions. Histopathologically, necrosis was seen in only D2/3 and G3 tumors. Microvascular invasion was seen in all grades. Metastasis occurred in all grades of primary NEN and the grades of the metastatic tumors and their corresponding primary tumors were similar in 67% of the cases. Of the 79 patients, 36 had at least one follow up visit with a median duration of follow up of 8.5 mo (range: 1-50 mo). This study did not show any impact of the grade of tumor on the short term clinical outcome of these patients.

CONCLUSION

Liver biopsy is an important tool for clinicopathological characterization and grading of NEN, especially when the primary is not identified. Eighty-seven percent of the patients had multifocal liver lesions irrespective of the WHO grade, indicating a higher stage of disease at presentation. Follow up duration was inadequate to derive any meaningful conclusion on long term outcome in our study patients.

Key words: Liver; Neuroendocrine neoplasms; Ki-67; Gastroenteropancreatic neuroendocrine neoplasms; Metastasis; Microvascular invasion

Core tip: Neuroendocrine neoplasms (NEN) in liver are commonly metastatic. The clinicopathological features of NEN diagnosed on liver samples were analyzed and graded applying World Health Organization (WHO) 2010 classification of gastroenteropancreatic NEN. A marked male preponderance was noted in all WHO grades except for G2 tumors, wherein a slight female predilection was seen. Necrosis was noted only in higher grade tumors. Most patients had multifocal liver lesions favoring metastasis and higher stage of disease at presentation. Follow up duration was inadequate to derive any meaningful conclusion on long term outcome in our study patients.

Burad DK, Kodiatte TA, Rajeeb SM, Goel A, Eapen CE, Ramakrishna B. Neuroendocrine neoplasms of liver - A 5-year retrospective clinico-pathological study applying World Health Organization 2010 classification. *World J Gastroenterol* 2016; 22(40): 8956-8966 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i40/8956.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i40.8956

INTRODUCTION

Neuroendocrine neoplasms (NEN) are tumors arising from the neuroendocrine cells which are distributed throughout the body[1,2]. They commonly originate from gastrointestinal tract, lungs and pancreas and rarely from other sites such as gall bladder, thymus, testes and ovaries[3-6]. NEN are the second most common tumors to metastasize to liver after adenocarcinomas[7,8]. NEN in the liver are usually metastatic and primary tumors are rare[9-13]. Liver is the second most common site of metastatic NEN following lymph nodes[5]. Amongst the metastatic NEN in liver, those originating from gastroenteropancreatic (GEP) region are more common due to spread *via* the portal vein[14].

The GEP NEN have been classified by the World Health Organization (WHO) 2010[15] into 3 (G1-G3) grades based on mitotic activity and Ki-67/MIB-1 proliferation index. These are G1: mitotic count < 2/10 high power fields (hpf) and/or Ki-67 index ⩽ 2%, G2: mitotic count 2-20/10 hpf and/or Ki-67 index 3%-20% and G3: mitotic count > 20/10 hpf and/or Ki-67 index > 20%. If the mitotic count or Ki-67 proliferation index points to different grades, a higher grade has to be given[15,16]. Some studies have shown discordance between mitotic count and Ki-67 index[17,18] in some cases. They have shown that the grade discordant tumors with mitotic count of G1 and Ki-67 index of G2 behave worse than grade concordant tumors[17,18].

Targeted biopsy of the liver lesion is often required to confirm the diagnosis of neuroendocrine neoplasm, as often the primary may not be identified.

Utility of WHO 2010 grading of NEN in liver biopsies has not been carried out and hence, we undertook this study to pathologically characterize these cases and to correlate with clinical findings.

MATERIALS AND METHODS

This study included all cases of NEN involving the liver diagnosed in the Department of Pathology, from January 2011 to December 2015. The data was collected from Pathology online data base. A total of 82 cases were available. Of the 82 cases, 3 had only cytology smears without cell block material and hence were excluded from the study. The remaining 79 cases included liver biopsies (59), resection specimens (5), cytology smears with cell block (5) and referred slides and blocks (10). Of the 59 liver biopsies, 58 were ultrasound (US) guided and, 1 was a per-operative wedge biopsy sample. Of the 5 resection specimens, 3 were localized segmentectomy specimens and the remaining 2 were left lateral segmentectomies. All the 5 cytology cases were US guided. Of the 10 cases of referred slides and blocks, 6 were US guided

Table 1 Distribution of known primary sites *n* (%)

Site of primary	Biopsy proven (*n* = 18)	Lesion-on radiology (*n* = 27)	Total
Pancreas	6	16	22 (49.0)
Lung	4	4	8 (18.0)
Duodenum	1	3	4 (8.8)
Rectum	2	1	3 (6.6)
Ileum	2	-	2 (4.4)
Esophagus	1	-	1 (2.2)
Cecum	-	1	1 (2.2)
Gall bladder	1	1	2 (4.4)
Anterior mediastinum	-	1	1 (2.2)
Kidney	1	-	1 (2.2)

biopsies, 2 were per-operative wedge biopsies and the remaining 2 were resections.

One resection (left lateral segmentectomy) case included in this study was from a 40 year old lady diagnosed with neuroendocrine neoplasm by biopsy in 2009, who received 6 cycles of chemotherapy and metaiodobenzylguanidine (MIBG) ablation prior to resection.

The histopathological features and immunohistochemistry details in 79 cases were analyzed. Based on WHO 2010 classification of GEP NEN, all cases were graded as G1, G2 or G3. In this study, we assigned 2 more categories which included D1/2 (discordant 1/2) and D2/3 (discordant 2/3). The D1/2 grade tumors had a mitotic count of G1 and Ki-67 index of G2. The D2/3 tumor had a mitotic count of G2 and Ki-67 index of G3. We did not have any cases wherein the mitotic count was of a higher grade and Ki-67 index of a lower grade.

The relevant clinical and radiological findings and the follow up details which were available till the end of the study period (December 2015) were collected.

Continuous data was described as number, mean, median, minimum and maximum; and categorical data was described as number with percentage. Categorical data was compared with chi-square test. A two sided *P* value of < 0.05 was considered statistically significant. All statistical analyses were done using SPSS software version 17.0.

This study was approved by Institutional Review Board.

RESULTS

Of the 79 patients, 71 were of Indian origin, 7 from Bangladesh and 1 from Srilanka. Overall, there was a male preponderance (male:female = 53:26) in this study. G2 tumors showed a slight female predilection (M:F = 7:9) as compared to the other grades (M:F = 46:17).

The median age at presentation was 47 years (range 10-82 years). Fifty-six percent of the cases were seen between 5th to 6th decade.

The study patients had a delay of 3 mo (0-24 mo; median, range) from their first symptoms to their final diagnosis at hospital. The most common presentation was abdominal pain (81%) followed by loss of weight and appetite (41%), altered bowel habits (14%) and mass per abdomen (9%). In 9% of the patients, the tumor was incidentally detected.

A clinical diagnosis of malignancy was given in 77% of the patients.

In 18 patients, the primary site was identified by biopsy and in another 27 patients, a probable primary lesion was identified on radiological examination alone. In both the groups, the most common primary site was pancreas (Table 1). In the remaining 34 patients, a definite primary site could not be identified.

In this study we encountered three interesting cases. The first case was a 46 year old male presented with 2 years duration of pain in right hip and lower limbs, acromegaly and erectile dysfunction in 2009. Based on radiological, serological and pathological examination, he was diagnosed with multiple endocrine neoplasia type 1 with pituitary macroadenoma, insulinoma, gastrinoma, bilateral inferior parathyroid adenomas, primary hyperparathyroidism, thymic carcinoid and adrenal adenoma. In 2012, computed tomography (CT) of the abdomen revealed a 2.5-cm lesion in segment 3 of liver along with multiple pancreatic lesions. The patient underwent a distal pancreatectomy with splenectomy and resection of segment 3 of liver in 2014, which on pathological examination confirmed neuroendocrine neoplasm of WHO grade 2, in both pancreas and in liver. The tumor was multifocal in pancreas.

The second case was a 22 year old male presented with left flank pain of 2 mo duration in 2013. CT scan showed a 10-cm lobulated mass in the left kidney and a diagnosis of renal cell carcinoma was suspected. Patient underwent left nephrectomy and histologically, diagnosed as large cell neuroendocrine carcinoma. On follow-up, 2 years later, patient was detected to have multiple hypodense lesions in the liver, in segments 6, 4a, and 2, and the largest measured 1.7 cm. An US guided biopsy confirmed metastatic neuroendocrine neoplasm, WHO grade 2.

The third case was a 40 year old female who presented with jaundice, loss of weight and appetite, lower limb weakness and abdominal distension of 2 years duration. She was recently detected to have hypertension. Blood investigations revealed hypokalemia, hypomagnesaemia and elevated serum adrenocorticotropic hormone (ACTH) and cortisol levels. In view of the above findings, she was diagnosed with ACTH dependent Cushing syndrome. CT scan revealed multiple liver lesions in both lobes, largest measuring 2.8 cm. An US guided biopsy of liver lesion was diagnosed as neuroendocrine neoplasm, WHO grade 2.

Radiological findings

Radiological findings were available in all 79 cases. In

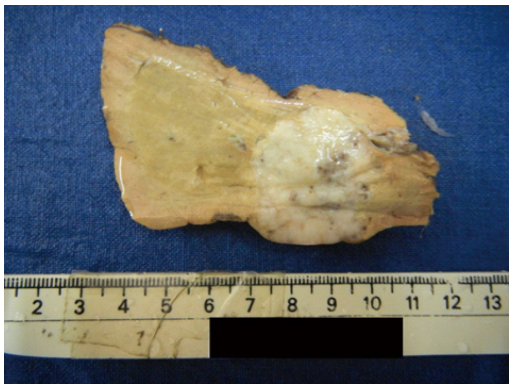

Figure 1 Liver with a fairly circumscribed tumor with a firm grey white cut surface.

69 cases (87%), the patients had multiple liver lesions (1.8 cm to 15.9 cm in maximum dimension) involving both the left and the right lobes. Remaining 10 patients (13%) had a single lesion (2.7 cm to 12.5 cm in maximum dimension), 4 of which were in the left lobe and 6 in the right lobe. A radiological diagnosis of metastases was made in 66 (83.5%) cases, hepatocellular carcinoma in 5 (6.3%), hemangioma in 1 (1.3%) and non-neoplastic etiology in 7 (8.9%), which includes 4 cases diagnosed as abscess.

Pathological findings

Of the seven liver resections in our study, five were available for gross examination and the remaining two were slides and blocks. Of the five, 3 were segmental resection specimens of segment 3, 6 and 8 respectively and the remaining 2 were left lateral segmentectomies. All resection specimens except one left lateral segmentectomy specimen had a single tumor nodule, ranging in size from 2.5 cm to 7.5 cm with a firm grey white to yellow cut surface (Figure 1). Focal area of hemorrhage was seen in one case. There was no evidence of necrosis or gross vascular invasion. The surrounding liver parenchyma was normal. One left lateral segmentectomy specimen following chemotherapy showed 2 tumor nodules, measuring 6.5 cm and 0.7 cm respectively. The cut surface of the tumor was firm grey white with focal areas of necrosis amounting to approximately 20% of the total tumor volume. There was no gross vascular invasion. The surrounding liver parenchyma was normal.

Based on WHO 2010 grading of the 79 tumors, 16 each were G1 and G2 and 18 were G3 tumors. Of the remaining 29 tumors, 13 were assigned to D1/2 grade and 16 were assigned D2/3 grade.

Histologically, the low grade tumors (G1, G2, D1/2) had a classical pattern of arrangement including nests, trabeculae, cords, ribbons, festoons, sheets, gyriform, pseudopapillary and acinar patterns (Figure 2A and C). The cells were round to polygonal with moderate to abundant amounts of eosinophilic granular cytoplasm and uniform to mildly pleomorphic nuclei with uniformly dispersed coarse chromatin and inconspicuous mitotic activity (Figure 3A).

The high grade tumors (G3, D2/3) showed nests and sheets of medium sized polygonal cells with mild to moderately pleomorphic nuclei with finely dispersed chromatin and scant to moderate amounts of eosinophilic cytoplasm. There was increased mitotic and apoptotic activity (Figures 2E and 3C).

Six cases had morphology consistent with small cell carcinoma with sheets and nests of polygonal cells displaying moderate nuclear pleomorphism, moulding, overlapping and increased mitotic and apoptotic activity.

The post chemotherapy and MIBG ablation resection specimen showed presence of occasional peritumoral non necrotizing epithelioid cell granulomas, probably reactive. Special stains for acid fast bacilli and fungal organisms were negative. None of the other 78 cases showed granulomas.

There was one interesting case of a 63 year old female with multiple liver nodules who underwent an US guided liver biopsy. Histologically, the tumor was composed of sheets and closely packed clusters of polygonal cells with eccentrically placed mild to moderately pleomorphic nuclei and abundant amounts of pale eosinophilic cytoplasm, resembling signet ring cells (Figure 4A). Occasional cells contained intracytoplasmic mucin droplets (Figure 4B). This case was diagnosed as signet ring cell neuroendocrine neoplasm.

Necrosis was identified in 12 cases and all were biopsies. Six of these were D2/3 grade and the remaining 6 were G3 grade. In 9 cases, it was present in small foci, while the remaining 3 showed extensive areas of necrosis (1 was G3 and 2 were D2/3). There was no necrosis in any of the resection cases except in the post chemotherapy case which showed necrosis and hyalinization, amounting to 20% of the entire tumor volume. However, there was no necrosis in the initial pre chemotherapy biopsy.

Microvascular invasion (MVI) was seen in 17 cases (4 resection cases and 13 biopsies). Of these 17 cases, 3 were G1, 4 G2, 6 G3 and 4 D2/3 grade tumors. In all the cases, tumor emboli were present in thin walled vascular channels and/or within sinusoids.

MVI was seen more frequently in high grade (G3 and D2/3) tumors (59%) when compared to low grade (G1 and G2) tumors (41%). However, it was not statistically significant. As most of these cases were biopsies, this increased frequency is most probably a chance finding.

We also looked at the presence of MVI in the 18 proven primary cases (12 resection, 4 biopsies and 2 cytology with cell block material). MVI was seen in 10 of 12 resection cases and none of the biopsied cases.

Status of nodal disease was also noted. Of the 79 patients, 7 had biopsy proven nodal metastasis and 35 had significant nodes on radiological examination. The remaining 37 patients did not have any significant

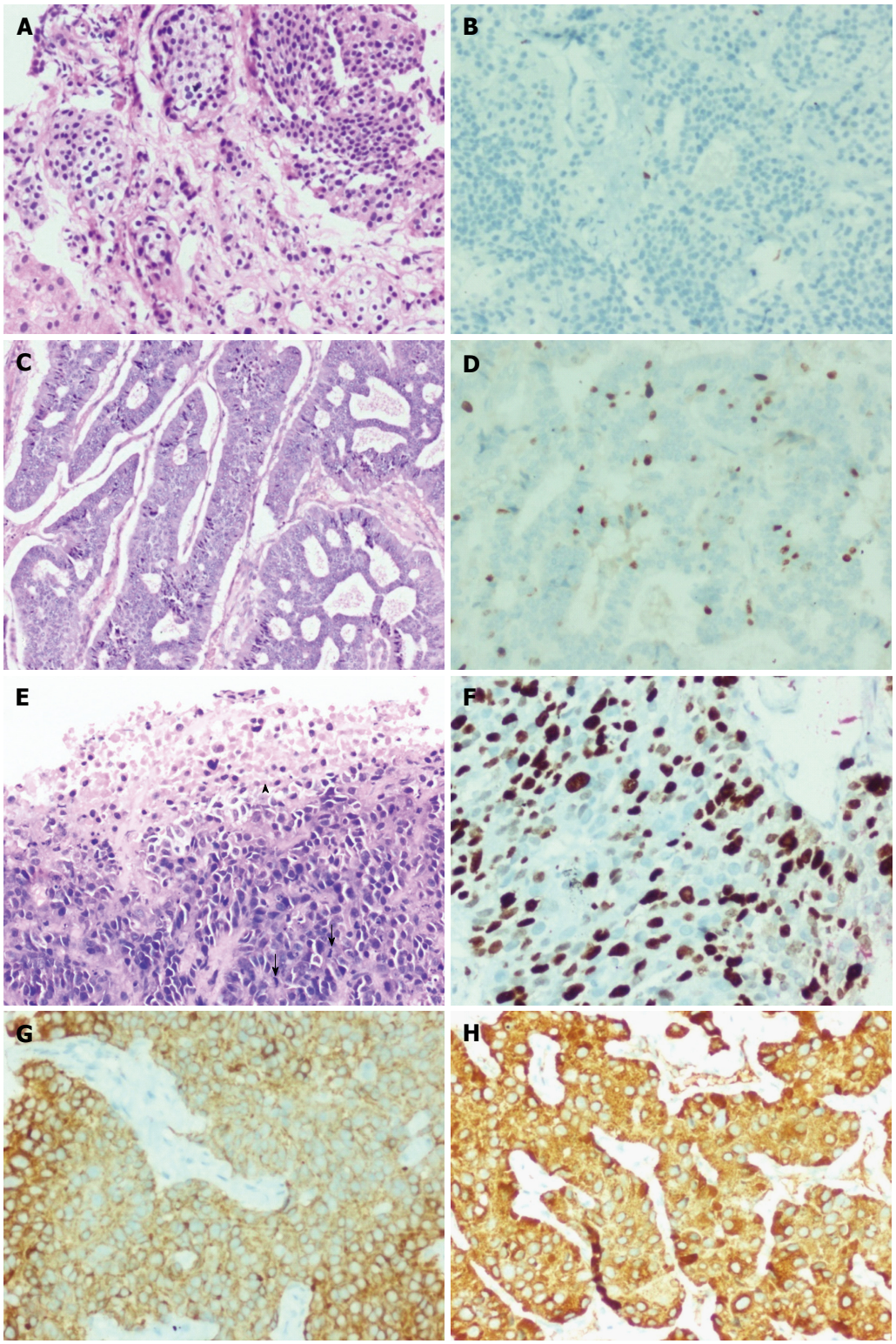

Figure 2 G1-G3 neuroendocrine neoplasms. A: G1 - tumor with trabecular and nested pattern (H&E × 200); B: Tumor cells displaying Ki-67 index of < 1% (immunohistochemistry × 200); C: G2 - tumor with gyriform and festooning patterns and the tumor cells display mildly pleomorphic nuclei with coarse stippled chromatin (H&E × 200); D: Tumor cells displaying Ki-67 index of approximately 16% (immunohistochemistry × 200); E: G3 - tumor cells with pleomorphic and hyperchromatic nuclei displaying brisk mitotic (arrow) and apoptotic activity and focal necrosis (arrow head) (H&E × 200); F: Tumor cells displaying Ki-67 index of approximately 80% (immunohistochemistry × 200); G: Tumor cells displaying diffuse cytoplasmic positivity for synaptophysin (immunohistochemistry × 200); H: Tumor cells displaying diffuse cytoplasmic positivity for chromogranin (immunohistochemistry × 200).

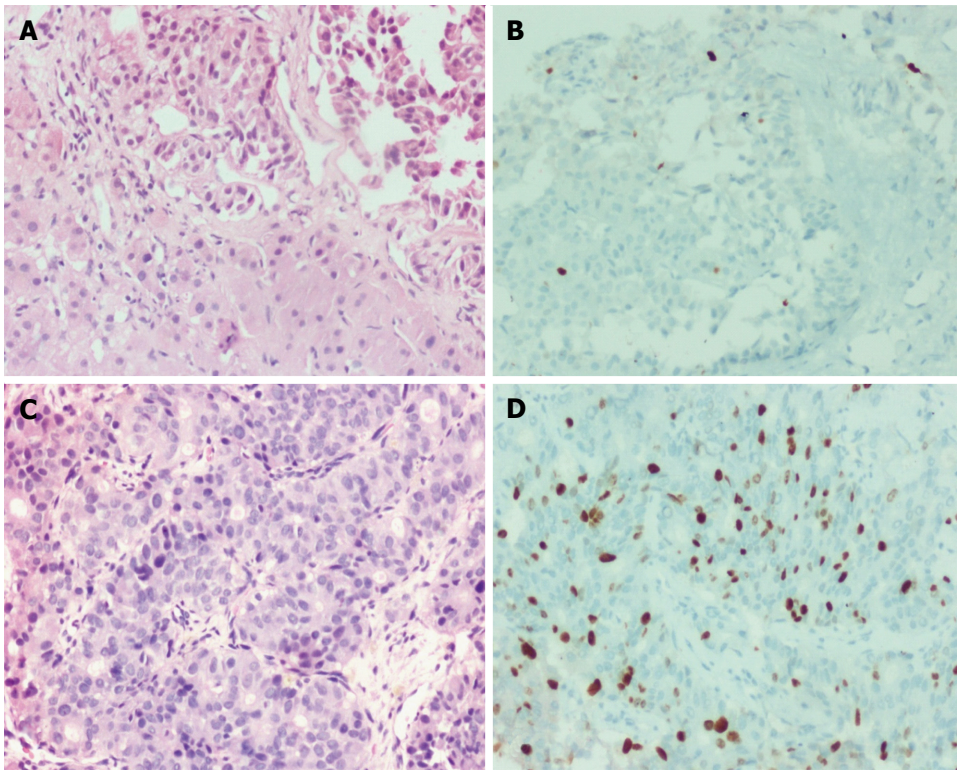

Figure 3 D1/D2 and D2/D3 neuroendocrine neoplasms. A: D1/2 - liver biopsy showing clusters of tumor cells with uniform nuclei and no conspicuous mitotic activity (H&E × 200); B: Tumor cells displaying Ki-67 index of approximately 5% (immunohistochemistry × 200); C: D2/D3 shows tumor cells arranged in trabeculae and focal acinar pattern with mildly pleomorphic nuclei (H&E × 200); D: Tumor cells displaying Ki-67 index of approximately 30% (immunohistochemistry × 200).

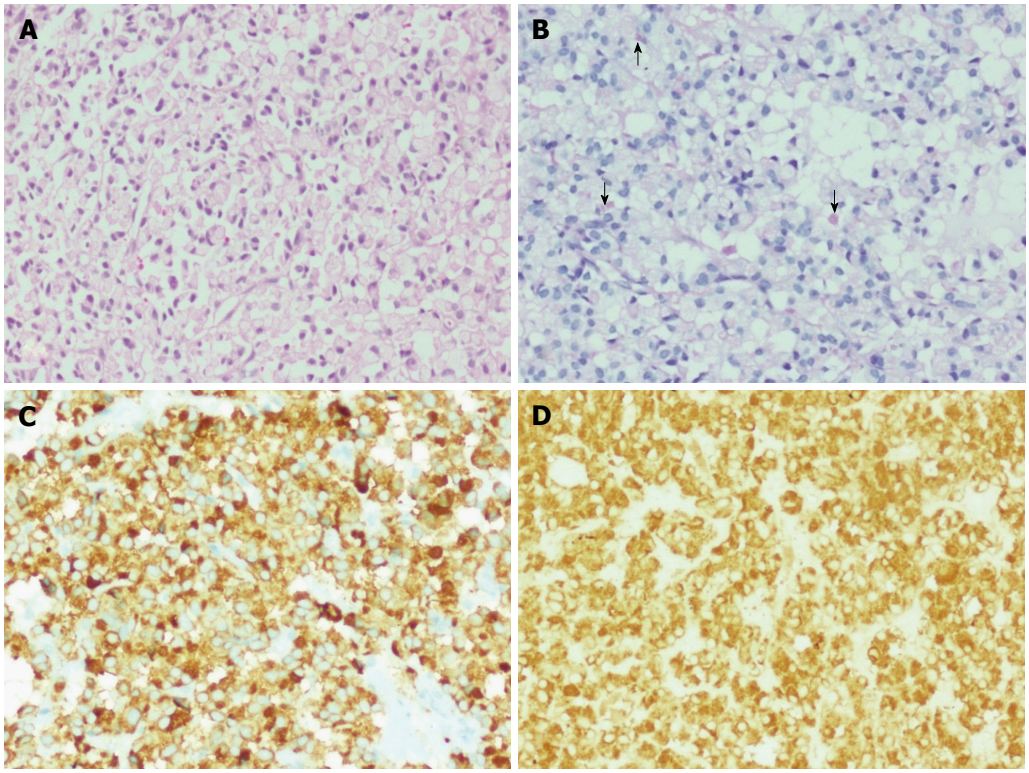

Figure 4 Signet ring cell neuroendocrine neoplasm, G2. A: Tumor cells arranged in sheets and composed of polygonal cells with abundant clear to vacuolated cytoplasm and eccentrically placed uniform nuclei (H&E × 200); B: Occasional tumor cells containing pale staining cytoplasmic mucin (arrows) (PAS-D × 200); C: Tumor cells displaying diffuse cytoplasmic positivity for synaptophysin (immunohistochemistry × 200); D: Tumor cells displaying diffuse cytoplasmic positivity for chromogranin (immunohistochemistry × 200).

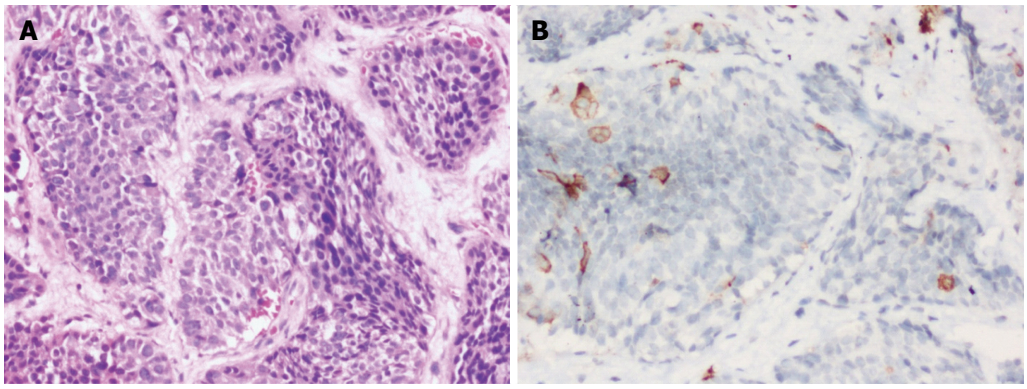

Figure 5 Adrenocorticotropic hormone producing neuroendocrine neoplasm, G2. A - Tumor cells arranged in nests and islands. (H&E × 200); B: Occasional tumor cells displaying cytoplasmic positivity for adrenocorticotropic hormone (immunohistochemistry × 200).

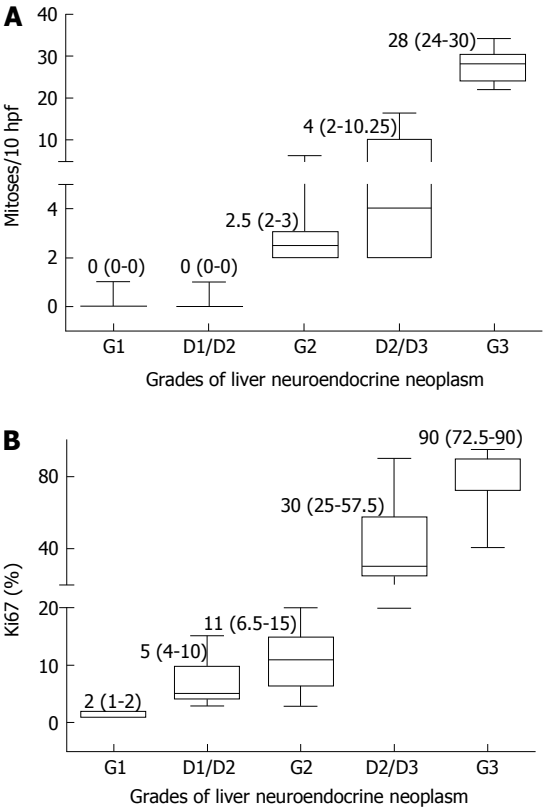

Figure 6 Correlation between the mitotic index and Ki67 index and the World Health Organization grading in liver neuroendocrine neoplasms. A: Box-plot showing the median, interquartile range and range of the mitotic index for each World Health Organization (WHO) grade of tumor; B: Box-plot showing the median, interquartile range and range of the Ki67 index for each WHO grade of tumor.

lymph node enlargement.

Immunohistochemistry

Immunostaining for two markers - synaptophysin and chromogranin were carried out in all 79 cases. Synaptophysin (Figure 2G) was positive in 77 and chromogranin (Figure 2H) in 75 cases, respectively.

In all 6 cases which were negative for either synaptophysin or chromogranin, CD 56 was found to be positive.

Pancytokeratin immunostaining done in 34 cases with high grade (G3:18, D2/3:16) neoplasms, showed positive staining in all except two cases with G3 tumor.

Immunostaining for thyroid transcription factor (TTF-1) was carried out in 9 cases. Six of these had morphology of small cell carcinoma and the remaining three had a lung lesion on radiology. Five out of 6 cases of small cell carcinoma type were TTF1 positive and 2 of these cases had a lung mass indicating a lung primary. One of the 3 cases with radiologically detected lesion in the lung was TTF1 positive.

The patient with signet ring cell NEN showed the tumor cells to be positive for CK7, synaptophysin (Figure 4C) and chromogranin (Figure 4D) and negative for CK20 and CDX2. Ki-67 proliferation index was 15%.

The patient with Cushing's syndrome showed neuroendocrine neoplasm with patchy cytoplasmic positivity within tumor cells for ACTH on immunohistochemistry (Figure 5A and B).

Ki-67 proliferation index in various grades of tumors are shown in Figures 2B, 2D, 2F, 3B and 3D. The medians of mitotic and Ki-67 proliferation indices are shown as box plots (Figure 6A and B). We did not have any cases, wherein mitotic count was of higher grade and Ki-67 index was of lower grade.

We also compared the WHO grading of tumors metastatic to liver with their biopsy proven 18 primary tumors (Figure 7). The grades of the metastatic tumors and the corresponding primary tumors were similar in 12 /18 cases (67%), lower in 5/18 (28%) and higher in 1/18 (5%) cases.

We noticed that metastasis can occur in NEN irrespective of whether the tumor is a low grade or high grade.

Comparison of G1 tumors (16 cases) with discordant D1/2 tumors (13 cases) revealed slightly lower median age at presentation in (47 years *vs* 52 years) and presence of MVI (19% *vs* 0%) in the former. However, there was no difference in sex distribution between the two groups.

Comparison of G2 tumors (16 cases) with discordant

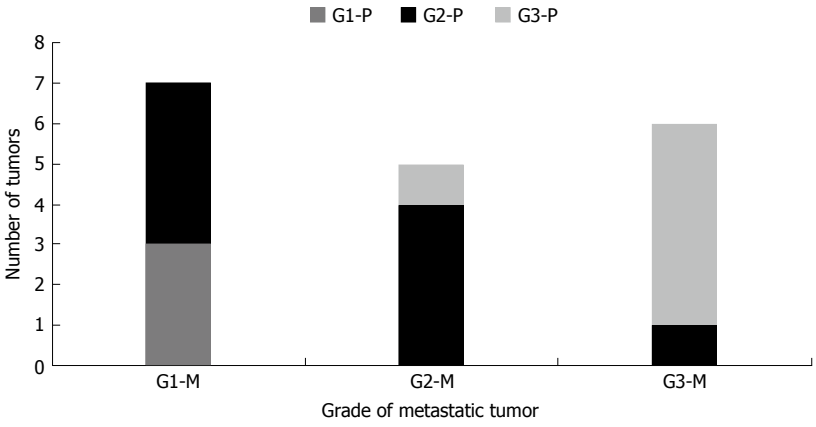

Figure 7 Shown here are tumor pairs in which the World Health Organization grade of the primary and metastatic tumors could be compared. G1-P, G2-P and G3-P refer to the World Health Organization (WHO) grade of the primary tumor, while G1-M, G2-M and G3-M refer to WHO grade of the metastatic tumor.

D1/2 tumors (13 cases) also revealed slightly lower median age at presentation (47 years *vs* 52 years), presence of MVI (25% *vs* 0%) and female predilection (56% *vs* 23%) in the former. Necrosis was not seen in any case in both grades.

Comparison of discordant D2/3 tumors (16 cases) with G2 tumors (16 cases) showed a slightly higher median age of presentation (51 years *vs* 47 years), male predilection (75% *vs* 44%) and presence of necrosis (25% *vs* 0%) in the former. MVI was seen in 25% of cases in both grades.

There was no statistically significant difference in any of these features between various groups.

Comparison of concordant G3 tumors (18 cases) with discordant D2/3 tumors showed no difference in age of presentation, sex distribution or presence of necrosis or MVI.

Treatment and follow-up

Forty (51%) of the 79 study patients were offered specific mode(s) of therapy - 9 (11.4%) underwent surgical resection which were either only liver resection (3 cases), both liver and primary tumor resection (2 cases; one with right hemicolectomy and the other with distal pancreatectomy) or only primary tumor resection (4 cases; 2 distal pancreatectomies, 1 lung resection and 1 subtotal gastrectomy). Thirty-three (43%) had chemotherapy and 7 (9%) had therapeutic 131I-MIBG therapy. 3 patients received a combination of chemotherapy and MIBG therapy, 4 patients underwent surgery in combination with chemotherapy and 1 patient received all three. Only 12 patients (Grade 1:4, Grade 2:4, Grade D2/D3:2, Grade 3:2) were deemed as cured after the initial therapy. There was no association of grade of disease and curative intent/achievement.

Of the 79 study patients, 36 patients had at least one follow up visit after diagnosis of neuroendocrine neoplasm. The median duration of follow up was 8.5 mo (range: 1-50 mo). During the follow up, five (Grade 3:2, Grade 2:2, Grade D1/D2:1) of the initial 12 cured patients suffered recurrence (median duration: 32 mo, range: 13-33 mo). Two patients (Grade 1:1, Grade D1/D2:1) showed continuous downhill course. Rest 29 followed patients (Grade 1:6, Grade D1/D2:5, Grade 2:5, Grade D2/D3:9, Grade 3:4) remained stable till the last follow up. One patient died (Grade D2/D3) within 10 d after the diagnosis.

DISCUSSION

To the best of our knowledge, this is the largest study describing detailed histological findings and relevant clinical data of 79 patients with hepatic NEN, metastatic in most cases.

Metastases from NEN are much more common than primary NEN in liver[9-13]. In a study of 393 digestive neuroendocrine tumors, only 5% had a primary neuroendocrine neoplasm in liver[19]. In a study of 13715 neuroendocrine tumors, liver was the second most common site of metastasis after lymph nodes[5].

In our study, pancreas was the most common site of primary tumor metastasizing to liver accounting for 49% of the cases followed by GIT (24.4%) and lungs (18%). Other studies have also reported that pancreas to be the most common primary site followed by rectum, stomach and ileum amongst the GEP NEN metastasizing to liver[20-22]. Begum *et al*[23] have shown that in their study of 2009 cases of GEP NEN, pancreas was the most common site accounting for 34.2% of cases followed by midgut (5.8%), gastric (6.5%), colon (6.9%) and duodenum (4.8%). Dromain *et al*[24] have shown that small bowel as the common primary site in their study (43%) followed by pancreas (25%), lung (15%), colon (5%), thymus (2%) and unknown primary (10%).

In our study 69 (87%) patients had multiple liver lesions involving both lobes of liver, which is similar to the study done by Niederle *et al*[25] where multiple liver lesions were seen in 92% of their cases.

It has been shown that the primary hepatic NEN to be predominantly a single lesion when compared

to metastatic NEN[26]. In our study, a single lesion was identified in 10/79 cases (13%), of which a biopsy proven primary was confirmed in 7 cases and probable primary was identified by radiology in 2 and in one case, no primary was detected.

In our study, males were twice more commonly affected than females. Male predilection has been shown in both metastatic and primary neuroendocrine tumors by Shen *et al*[26] and Shin *et al*[22] in their studies. However, some studies have reported almost similar incidence in males and females[2,5,17,20,27-29]. The median age at diagnosis was 47 years in this study which was slightly lower compared to other studies, which have reported from 55-62 years[2,5,20,23,26].

The most common presentation was abdominal pain (81%). A similar finding was reported by Chan *et al*[20] in their study on 126 GEP NEN.

Three interesting cases were encountered in this study. One case was a primary renal neuroendocrine carcinoma which is very rare[30,31] and the patient developed liver metastasis 2 years after nephrectomy.

Another case was signet ring cell neuroendocrine neoplasm which is also very rare and is characterized by the presence of cytoplasmic vacuoles that are negative for mucin stain and positive for cytokeratin[32-34].

The third case was a 40 year old female who was diagnosed with ACTH dependent Cushing syndrome with a concomitant neuroendocrine neoplasm, diagnosed on liver biopsy. Ectopic secretion of ACTH by NEN of liver is extremely rare and has been described[6,35].

In our study, necrosis was identified only in high grade tumors, either G3 or D2/3. However, McCall *et al*[18] have shown in their study of 297 G1 and G2 pancreatic NEN, necrosis in both grades, but the frequency was higher in G2 as compared to G1.

In our study, among the 18 pathology proven primary cases, liver metastases occurred irrespective of the grade of the tumor. In contrast, liver metastases were seen more often in grade 2 or 3 tumors in a study of gastrointestinal neuroendocrine tumors[36].

In our study, the grades of the primary and secondary tumors were similar in 67% of the cases and there was no difference in grade and metastatic potential. This was in contrast to the study by Shi *et al*[37], in which 65% of the patients with grade 1 primary tumor developed a higher grade (G2 or 3) liver metastasis. This could be explained partly by the fact that the Ki-67 index was determined on multiple resected tumors in a single patient in their study and the highest WHO grade was taken, indicating the importance of intertumoral heterogeneity. Our study involves mainly samples from a single lesion (91% cases) and hence intertumoral heterogeneity could not be assessed. Intratumoral and intertumoral heterogeneity in Ki-67 index has also been described in a study of metastatic NEN of liver by Yang *et al*[38].

Of the 79 study patients, 36 patients had at least one follow up visit after diagnosis of neuroendocrine neoplasm with a median duration of follow up of 8.5 mo (range: 1-50 mo). However, this duration was not adequate to derive any meaningful conclusion on long term outcomes in our study patients. Shen *et al*[26] have shown in their study on liver NEN, a better survival in low grade tumors as compared to high grade tumors. This could be due to the fact that this study included only liver resection cases but our study was done predominantly on biopsy samples and also due to lesser number of patients available for follow-up. Also, 87% of our patients had multifocal liver lesions irrespective of the WHO grade, indicating a higher stage of disease at presentation itself.

The limitations of the study were the following; lack of follow-up data on all patients included in this study and though most of the patients had multiple liver lesions, biopsy or cytology sample was obtained from only one lesion. Intratumoral and intertumoral heterogeneity in Ki-67 index also could not be determined, which has been described in few studies[37,38].

In conclusion, liver biopsy is an important tool for clinicopathological characterization and grading of NEN, especially when the primary is not identified. Most of the cases had multifocal disease indicating metastasis. However, a possibility of a primary hepatic neuroendocrine tumor should be kept in mind, especially when it is a single lesion and a definite primary is not identified after extensive workup.

COMMENTS

Background

Neuroendocrine neoplasms (NEN) in liver are commonly metastatic. In many cases, a primary cannot be identified and in some cases where primary is identified, the site may not be accessible for biopsy. In such scenarios, a liver biopsy is of great help to characterize these tumors.

Research frontiers

The authors have studied the clinicopathological features of NEN in liver and graded them according to the World Health Organization (WHO) 2010 grading of gastroenteropancreatic NEN.

Innovations and breakthrough

The grades of the metastatic tumors and the corresponding primary tumors were similar in 67% of the cases. Few interesting and rare cases were also identified.

Applications

Liver biopsy is an important tool for clinicopathological characterization and grading of NEN, especially when the primary is not identified.

Terminology

NEN are tumors arising from the neuroendocrine cells which are distributed throughout the body. The WHO 2010 grades these tumors in to grade 1, 2 and 3 based on mitotic activity and Ki-67 proliferation index.

Peer-review

The authors provide a comprehensive study of NEN of liver that includes 79 patients, some interesting cases among them. Neuroendocrine neoplasm in the liver is not a common disease. According to the authors, this is the largest

study so far describing detailed histological findings and relevant clinical data of patients with hepatic NEN. The authors had drawn some valuable conclusions for this disease.

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P- Reviewer: Corrales FJ, Peck-Radosavljevic M, Wu SL
S- Editor: Gong ZM **L- Editor**: A **E- Editor**: Zhang FF

World Journal of Gastroenterology

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DOI: 10.3748/wjg.v22.i40.8967

World J Gastroenterol 2016 October 28; 22(40): 8967-8977
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

ORIGINAL ARTICLE

Clinical Trials Study

Effects of an oral iron chelator, deferasirox, on advanced hepatocellular carcinoma

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Author contributions: Saeki I and Yamamoto N contributed equally to this work; Saeki I, Yamamoto N and Yamasaki T analyzed the data; Saeki I, Yamamoto N and Yamasaki T wrote the paper; Saeki I, Yamasaki T, Iwamoto T, Hidaka I, Ishikawa T and Uchida K performed the clinical experiments; Yamamoto N, Takami T, Maeda M, Fujisawa K, Matsumoto T and Tani K performed the basic experiments; Tani K and Sakaida I contributed reagents/materials/analysis tools.

Supported by Grants-in-Aid for Scientific Research from the Japan Society for the Program of Science, No. 23590978 and No. 16H05287; the Strategic Research Promotion Program from Yamaguchi University.

Institutional review board statement: This study (H25-148) was approved by the Institutional Review Board of Yamaguchi University Hospital. The study protocol was designed according to the principles of the 1975 Declaration of Helsinki.

Clinical trial registration statement: This study is single arm, non-randomized, open-labeled study. The trial was registered online (http://www.umin.ac.jp/) (UMIN 000013451).

Informed consent statement: Written informed consent was obtained from all patients prior to enrollment.

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

Data sharing statement: No additional data are available.

Manuscript source: Invited manuscript

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Received: June 28, 2016
Peer-review started: June 29, 2016
First decision: August 19, 2016
Revised: September 6, 2016
Accepted: September 28, 2016
Article in press: September 28, 2016
Published online: October 28, 2016

Abstract

AIM

To evaluate the inhibitory effects of deferasirox (DFX) against hepatocellular carcinoma (HCC) through basic and clinical studies.

METHODS

In the basic study, the effect of DFX was investigated in three hepatoma cell lines (HepG2, Hep3B, and Huh7), as well as in an N-nitrosodiethylamine-induced murine HCC model. In the clinical study, six advanced HCC patients refractory to chemotherapy were enrolled. The initial dose of DFX was 10 mg/kg per day and was increased by 10 mg/kg per day every week, until the maximum dose of 30 mg/kg per day. The duration of a single course of DFX therapy was 28 consecutive days. In the event of dose-limiting toxicity (according to the Common Terminology Criteria for Adverse Events v.4.0), DFX dose was reduced.

RESULTS

Administration of DFX inhibited the proliferation of hepatoma cell lines and induced the activation of caspase-3 in a dose-dependent manner *in vitro*. In the murine model, DFX treatment significantly suppressed the development of liver tumors ($P < 0.01$), and significantly upregulated the mRNA expression levels of hepcidin ($P < 0.05$), transferrin receptor 1 ($P < 0.05$), and hypoxia inducible factor-1α ($P < 0.05$) in both tumor and non-tumor tissues, compared with control mice. In the clinical study, anorexia and elevated serum creatinine were observed in four and all six patients, respectively. However, reduction in DFX dose led to decrease in serum creatinine levels in all patients. After the first course of DFX, one patient discontinued the therapy. We assessed the tumor response in the remaining five patients; one patient exhibited stable disease, while four patients exhibited progressive disease. The one-year survival rate of the six patients was 17%.

CONCLUSION

We demonstrated that DFX inhibited HCC in the basic study, but not in the clinical study due to dose-limiting toxicities.

Key words: Liver tumor; Hepatocellular carcinoma; Advanced stage; Iron-chelator; Deferasirox

Core tip: There are currently no established second-line chemotherapies for advanced hepatocellular carcinoma (HCC) patients. Iron chelators exert antiproliferative effects in several cancers. We demonstrated the inhibition of HCC by deferasirox (DFX) in the basic study. However, the efficacy of DFX in our clinical study could not be verified due to dose-limiting toxicities. Although iron chelators have promising therapeutic potential, further examinations are necessary to establish their clinical applications.

Saeki I, Yamamoto N, Yamasaki T, Takami T, Maeda M, Fujisawa K, Iwamoto T, Matsumoto T, Hidaka I, Ishikawa T, Uchida K, Tani K, Sakaida I. Effects of an oral iron chelator, deferasirox, on advanced hepatocellular carcinoma. *World J Gastroenterol* 2016; 22(40): 8967-8977 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i40/8967.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i40.8967

INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common cancer and the second leading cause of cancer-related deaths worldwide[1]. Although recent advances in treatment techniques have improved the prognosis of this malignancy, the prognosis of advanced HCC patients, especially in the case of vascular invasion and/or extrahepatic spread, remains poor[2,3]. For such patients, the multikinase inhibitor sorafenib is recommended as the current standard therapy worldwide[4,5]. On the other hand, hepatic arterial infusion chemotherapy (HAIC) is one of the recommended treatments in Japan[5]. Sorafenib is generally used to treat patients with Child-Pugh A score, while HAIC is indicated for those with Child-Pugh A or B scores. Therefore, both HAIC and sorafenib are first-line chemotherapy options for those with Child-Pugh A score. On the other hand, the options available for those with Child-Pugh B score are either HAIC or systemic chemotherapy with the exception of sorafenib[6]. However, there are currently no established second-line chemotherapies for advanced HCC patients.

Iron is essential for a number of cellular metabolic processes, including DNA synthesis[7]. It is also required for the proliferation of cancer cells before initiation of DNA synthesis[8]. Iron chelators are commonly used for the treatment of iron-overload disease. Although iron chelators are not classified as anticancer drugs, they exert antiproliferative effects in several cancers, including HCC[9-12]. We previously reported that deferoxamine (DFO) can prevent both liver fibrosis and development of preneoplastic lesions in rats[13,14]. We also performed a pilot study of DFO for HAIC in advanced HCC patients for the first time, and demonstrated the efficacy of this chelator[15]. Hence, DFO therapy may be used as second-line chemotherapy owing to its therapeutic potential in patients with deteriorated liver function. However, DFO cannot be administered orally, thus limiting its clinical application. Recently, deferasirox (DFX), a newly developed oral iron chelator, was shown to exert a powerful antiproliferative effect in human hepatoma cell culture[12], and on hepatocarcinogenesis *in vivo*[16].

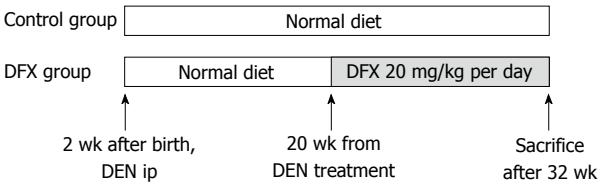

Figure 1 Protocol for the murine model of hepatocarcinogenesis. The model was induced by injection of 10 μg/g of DEN at 14 d of age. In the deferasirox (DFX) group, 20 mg/kg of DFX was administered orally for 3 mo and fed with normal diet. In the control group, the same amount of normal diet was administered. After 3 mo (at week 32), the mice were sacrificed and underwent autopsy examination.

We have also reported that DFX, like DFO, is able to prevent liver fibrosis and hepatocarcinogenesis in rats. In addition, we have shown that DFX can prevent the adverse effects of sorafenib[17]. Thus, DFX may represent a next-generation option for chemoprevention of HCC. However, there have been no *in vivo* or clinical studies of DFX against HCC. Therefore, the aim of this study is to evaluate the inhibitory effects of DFX against HCC, through both basic and clinical research.

MATERIALS AND METHODS

Basic research

Cell proliferation assay: Hepatoma cell lines (HepG2, Hep3B, and Huh7) were seeded at a density of 1.0 × 10^4 cells/well in a 96-well plate. Cell proliferation was measured using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay with CellTiter 96 AQueous One Solution Reagent (Promega, Madison, WI, United States). Cells were treated with DFX (0, 10, 20, 50, and 100 μmol/L) after 12 h, and were incubated for 24 h. The absorbance at 490 nm was measured to evaluate cell viability using a Bio-Rad plate reader (Hercules, CA, United States)[18].

Caspase-3 activity: Hepatoma cell lines were seeded at a density of 1.0 × 10^4 cells/well in a 96-well plate, and incubated in serum-free medium for 24 h. Then, the cells were treated with DFX (0, 10, 20, 50, and 100 μM) and incubated for another 24 h in serum-free medium. The activity of caspase-3 was determined using a caspase-3 colorimetric assay kit (MBL, Nagoya, Japan). This assay measured the cleavage of a specific colorimetric caspase substrate, DEVD-pNA, which releases *p*-nitroaniline (pNA). Free pNA produces a yellow color that was detected by a spectrophotometer at 405 nm[18].

Animals and experimental protocol: Animal care was performed in accordance with the animal ethics requirements of Yamaguchi University School of Medicine; the approval ID of the experimental protocol was 21-025. Seven-week-old female C57 BL/6 mice (20-30 g) were purchased from Nippon SLC (Shizuoka, Japan) and housed in a room under controlled temperature (25 ℃) and lighting (12-h light, 12-h dark) at the Animal Experiment Facility of Yamaguchi University School of Medicine.

Male mice received a single intraperitoneal injection of 10 μg/g body weight of N-nitrosodiethylamine (DEN) (Sigma-Aldrich Japan, Tokyo, Japan) at 14 d of age. Incidence of liver tumors was histologically evaluated five months after DEN injection[19]. The mice were divided into two groups (n = 10 per group): normal diet only (control group) and normal diet with DFX (DFX group) (Figure 1). At 20 wk after DEN injection, DFX (20 mg/kg per day) was administered orally for a period of 3 mo until week 32. Food intake of mice in each group was measured. To equalize the total food intake in all groups, additional food was not supplied until all food had been consumed.

Histology and immunohistochemical examination: Sections (3 μm thick) of the mouse liver were fixed in 4% paraformaldehyde (Muto; Tokyo, Japan) for 24 h and embedded in paraffin. The sections were processed for hematoxylin and eosin (H&E) staining. Tumor area of the liver in H&E stain was quantified using a Keyence BIOREVO BZ9000 microscope (Osaka, Japan) and was expressed as percentage of the total specimen area.

Real-time quantitative polymerase chain reaction: Expression of hepcidin, transferrin receptor 1, and hypoxia inducible factor-1α (HIF-1α) mRNA between tumor and non-tumor tissues was evaluated by real-time polymerase chain reaction (PCR) as described previously[17]. Briefly, RNA extraction was performed using an RNeasy Mini kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's protocol. The primers used were as follows:

Mouse hepcidin: sense (5′-AGAGCTGCAGCCTTTGCAC-3′),

antisense (5′-GAAGATGCAGATGGGGAAGT-3′);

Mouse transferrin receptor 1: sense (5′-GGTGATCCATACACACCTGGCTT-3′),

antisense (5′-TGATGACTGAGATGGCGGAA-3′);

Mouse HIF-1α: sense (5′-GCGTGCATGTCTAATCTGTTCC-3′),

antisense (5′-GATTCTGACATGCCACATAGCTC-3′);

Mouse β-actin: sense (5′-TGACAGGATGCAGAAGGAGA-3′),

antisense (5′-GCTGGAAGGTGGACAGTGAG-3′).

PCR amplification was performed in triplicate using the following cycle conditions: 40 cycles of 90 ℃ for 30 s, 55-60 ℃ for 45 s, and 72 ℃ for 1 min. Gene expression levels were analyzed using β-actin as the reference gene.

Mice survival and serum components: To analyze mice survival in the control (n = 10) and DFX (n = 9) groups, mice were fed normal diet and administered the same dose of DFX (20 mg/kg per day) for approximately one year. We used the Kaplan-Meier estimator of survival, and verified the survival function

against lifetime data.

Serum samples were obtained by eye puncture method at 32 weeks (n = 8 for control group, n = 5 for DFX group). In all experiments, serum total protein, total bilirubin, albumin, alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), blood urea nitrogen (BUN), and creatinine levels were measured using an analyzer for clinical chemistry (SPOTCHEM EZ SP-4430; Arkray, Kyoto, Japan).

In addition, we measured body weight of the mice at 32 wk.

Clinical research

Patients: The eligibility criteria for inclusion in this study were as follows: age > 20 years; Child-Pugh score A or B; leukocyte count > 2500/mm^3; platelet count > 5000/mm^3; hemoglobin level > 9 g/dL; prothrombin activity > 50%; total bilirubin < 3 mg/dL; serum creatinine < 2 mg/dL; unresectable HCC due to extensive, locally advanced disease, bilobar disease, extrahepatic metastasis, or vascular tumor thrombosis; refractory to chemotherapy; and Eastern Cooperative Oncology Group performance status of 0 or 1[20]. The exclusion criteria were as follows: severe complicating disease; concomitant malignancy; a history of allergy; pregnancy/lactation; a history of interstitial pneumonia; chronic respiratory failure; and severe general condition.

Six patients who were admitted to our hospital enrolled in this study between April 2014 and July 2015. The diagnosis of HCC was performed based on imaging results and elevated serum levels of alpha-fetoprotein and/or des-gamma-carboxy prothrombin.

This study (H25-148) was approved by the Institutional Review Board of Yamaguchi University Hospital, and written informed consent was obtained from all patients. The study protocol was conducted according to the principles of the 1975 Declaration of Helsinki. The trial was registered online (http://www.umin.ac.jp/) (UMIN 000013451).

Study design and treatment protocol: This study was designed as a dose-escalation trial in which patients received continuous oral administration of DFX at doses ranging from 5 to 30 mg/kg per day. The initial dose of DFX was 10 mg/kg per day, based on the minimal dose used for the treatment of iron-overload disease. If patients did not experience dose-limiting toxicity (DLT) that was beyond the grade 3 adverse event (AE) within one week, the dose of DFX was increased by 10 mg/kg per day every week, until it reached 30 mg/kg per day. On the other hand, if a serious clinical toxicity was observed, the dose of DFX was either decreased (grade 3 AE) or the treatment discontinued (grade 4 AE). Nevertheless, because renal dysfunction developed in the initial three cases, we reduced the DFX dose if the estimated glomerular filtration rate (eGFR) decreased to less than 50% of the baseline level.

A single course of DFX treatment consisted of DFX administration for 28 continuous days. After the treatment was suspended for one or two weeks and its safety was confirmed, DFX was administered at the maintenance dose (5-15 mg/kg per day) in the outpatient department. Patients continued treatment until disease progression, withdrawal of consent, or in cases of intolerability. Toxicity was graded using the Common Terminology Criteria for Adverse Events v.4.0 (CTCAE v.4.0)[21]. Tumor measurements were performed at baseline and at the end of every course using dynamic computed tomography or magnetic resonance imaging, and the evaluation of the response to the treatment was classified according to the mRECIST guideline[22]. When repeated DFX treatment was performed, the best response was considered for response evaluation. Survival time was defined as the interval between DFX administration and the last follow-up or death. The follow-up period ended on December 31, 2015. The primary endpoint was safety, and the secondary endpoint was tumor response and overall survival.

Statistical analysis

Statistical significance was assessed using student's t-test for biochemical and histological results, and the log-rank test for survival analysis. Human results are expressed as mean ± SD, differences with $P < 0.05$ were considered significant. All analyses were performed using the JMP ver. 10.0 software package (SAS Institute, Cary, NC, United States).

RESULTS

Basic research

Effect of DFX on hepatoma cell lines: Administration of DFX inhibited the proliferation of all hepatoma cell lines in a dose-dependent manner (Figure 2A). In addition, DFX also induced the activity of caspase-3 in a similar manner (Figure 2B).

Effect of DFX on tumor in murine model: Figure 3A (control group) and B (DFX group) show macroscopic images of tumor formation in mice liver. The total tumor area was significantly reduced ($P < 0.01$) in the DFX group compared with the control group (Figure 3C).

Effect of DFX on the expression of iron-related genes: Hepcidin, transferrin receptor 1, and HIF-1α mRNA expression levels in both tumor and non-tumor areas were significantly higher ($P < 0.05$) in the DFX-treated group than the control group (Figure 4A and C).

Effect of DFX on mice survival and serum components: Table 1 shows the levels of different serum

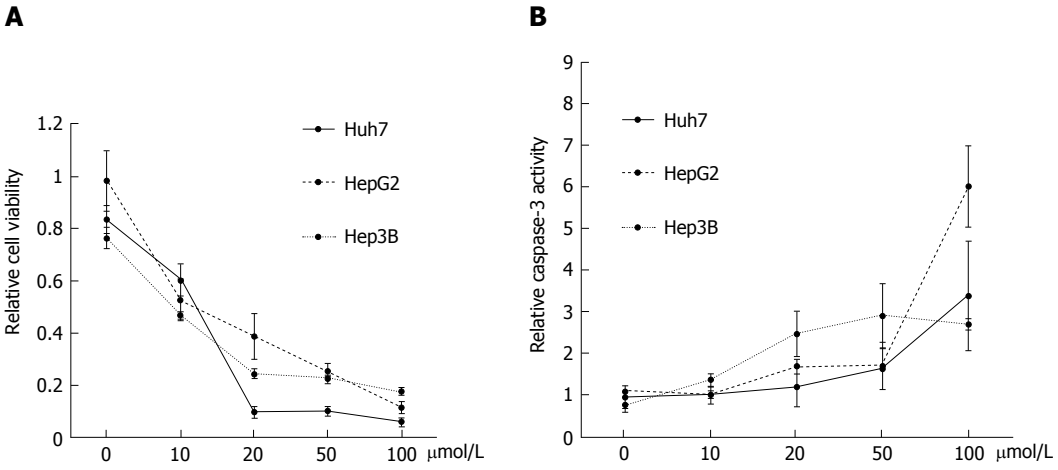

Figure 2 Antitumor effects of deferasirox in hepatoma cell lines. A: Deferasirox (DFX) exhibited antiproliferative effects against each cell line in a dose-dependent manner as revealed by MTT assay. Bars represent SD; B: Colorimetric assay of caspase-3 activity showing activation of caspase-3 by DFX in a dose-dependent manner. Bars represent SD.

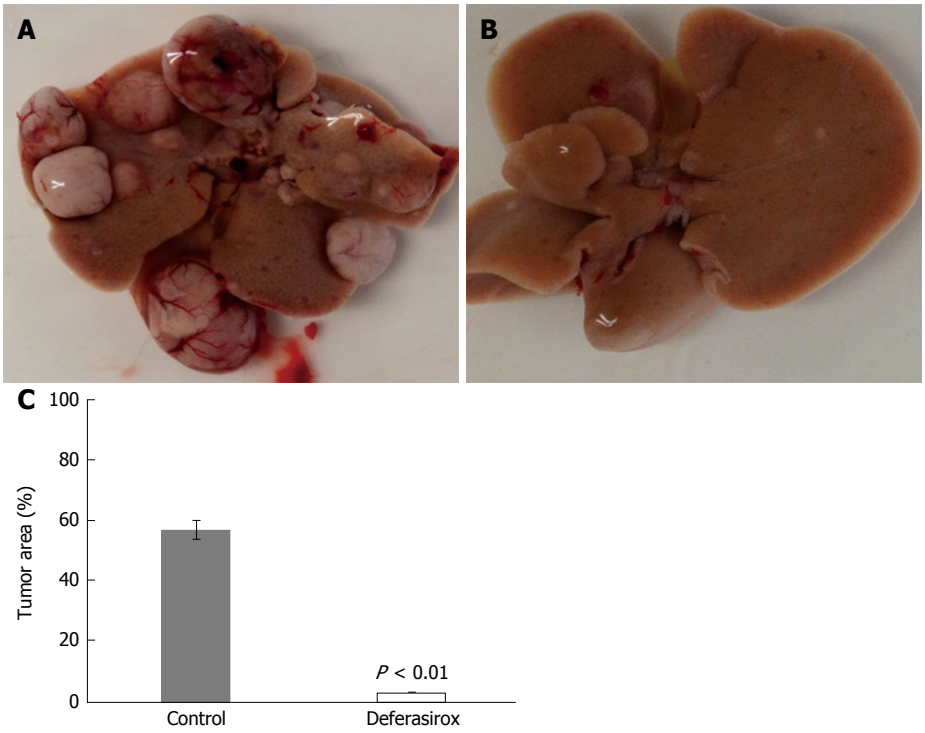

Figure 3 Inhibition of liver tumor formation by deferasirox. Macroscopic images of liver tumors in the (A) control and (B) deferasirox (DFX) groups. Evaluation of (C) tumor area percentage of the total specimen area in control and DFX-treated mice. Bars represent SD.

components in DFX and control groups. Mice in the DFX group had significantly lower level of serum ALT than those in the control group ($P < 0.05$). However, there were no significant differences in the levels of total protein, albumin, total bilirubin, AST, ALP, BUN, creatinine, and body weight between both groups. Liver dysfunction and renal dysfunction were not observed in the DFX group in this murine model.

Survival analysis of mice in the two groups over approximately one year showed that DFX-treated mice survived significantly longer ($P < 0.01$) than control mice (Figure 5).

Clinical research

Patient characteristics: The patients' characteristics are shown in Table 2. There were four male and two female patients with an average age of 70 years (range, 60 to 80). Five patients had hepatitis C virus infection, while one had hepatitis B virus infection. According to the Child-Pugh classification, half of the patients were classified as class A, and the other half as class B. The tumor stages were classified as Ⅲ ($n = 2$) and IVA

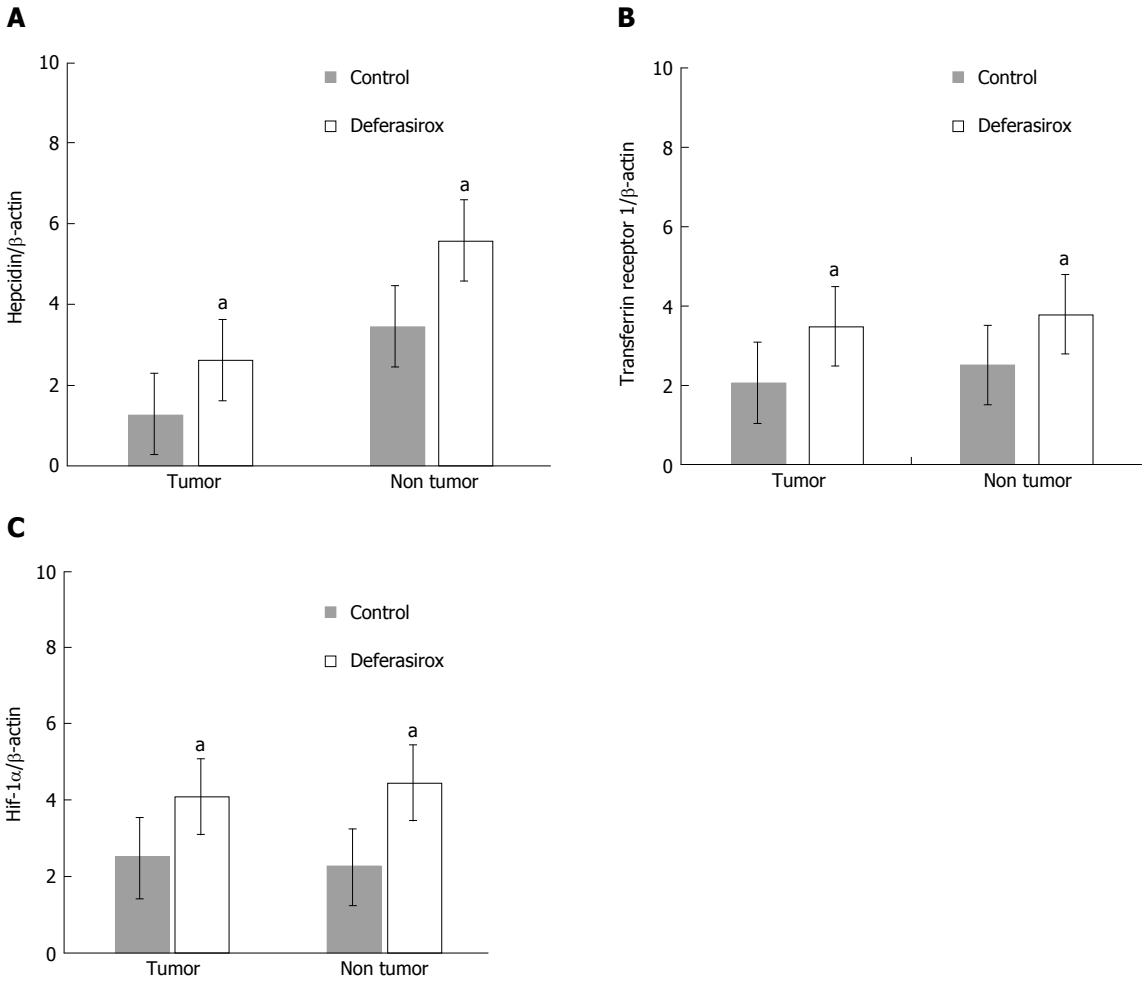

Figure 4 Regulation of iron-related gene expressions by deferasirox in a murine model. Real-time RT-PCR data of the expressions of (A) hepcidin, (B) transferrin receptor 1, and (C) HIF-1α in tumor and non-tumor tissues. Bars indicate SD. $^{a}P < 0.05$.

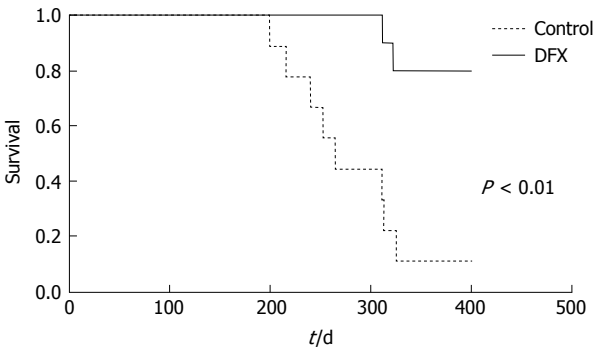

Figure 5 Cumulative survival rates of control and deferasirox-treated mice in a murine hepatocellular carcinoma model. Deferasirox (DFX)-treated mice showed significantly higher survival rate than control mice ($P < 0.01$).

($n = 4$), according to the Liver Cancer Study Group of Japan[23].

Safety and tolerability of DFX: Table 3 summarizes the adverse events observed during the trial. Although all patients showed renal dysfunction, their conditions improved with reduction in DFX dose (Figure 6A and B). Elevated creatinine levels observed in patients were classified into different AE grades; grade 3 in one patient, grade 2 in four patients, and grade 1 in one patient. Anorexia was observed in four patients (grade 2 in two patients, grade 1 in two patients). However, there were no treatment-related deaths.

DLT of DFX was observed in patients treated with high doses of the drug (≥ 20 mg/kg per day). Two patients experienced DLT at a dose of 30 mg/kg per day, and another four patients at 20 mg/kg per day. After the first course of DFX, all patients required dose reductions and one patient discontinued treatment due to intolerance associated with anorexia.

Tumor response and survival: We assessed tumor response to DFX in all patients except one who discontinued the treatment (Case 1). One patient exhibited stable disease (SD), and four patients exhibited progressive disease (PD) (Table 2). One patient who had multinodular HCCs without portal vein tumor thrombus (Case 3) maintained SD for eight months (Figure 7).

The one-year cumulative survival rate of the six patients was 17%, and the median survival time was 271 d. Five patients died of cancer-related disease,

Table 1 Comparison of body weight and serum data between deferasirox and control groups

	Body weight (g)	TP (g/dL)	Albumin (g/dL)	T-bil (mg/dL)	AST (IU/L)	ALT[a] (IU/L)	ALP (IU/L)	BUN (mg/dL)	CRE (mg/dL)
Control	40.7 ± 4.1	5.6 ± 0.4	2.8 ± 0.2	0.5 ± 0.1	95.8 ± 33.9	92.3 ± 43.1	175.1 ± 23.2	25.5 ± 2.9	0.4 ± 0.1
DFX	42.8 ± 4.7	5.2 ± 0.5	2.7 ± 0.1	0.6 ± 0.3	87.6 ± 20.1	29.5 ± 13.8	162.2 ± 35.9	30.2 ± 1.6	0.6 ± 0.1

Mean ± SD. [a]$P < 0.05$. DFX: Deferasirox; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; ALP: Alkaline phosphatase; BUN: Blood urea nitrogen; TP: Total protein; CRE: Creatinine.

Table 2 Patients' characteristics

Case	Sex	Age	Etiology	Child-Pugh score (Points)	Stage[1]	BCLC[2]	Maintenance dose (mg/kg per day)	Administration period (d)	Response	AFP (ng/mL)		DCP (mAU/mL)	
										Baseline	After 1 course	Baseline	After 1 course
1	M	80	HCV	B (7)	ⅣA	C	10	17	PD	1918	-	57	-
2	M	73	HBV	B (7)	Ⅲ	C	10	35	PD	127402	260627	73	61
3	M	72	HBV	B (8)	Ⅲ	B	5	248	SD	189	394	85	25
4	M	65	HBV	A (5)	ⅣA	C	10	28	PD	603	814	9995	16793
5	F	70	HCV	A (6)	ⅣA	C	15	29	PD	5580	8434	19958	15321
6	F	60	HCV	A (6)	ⅣA	C	15	28	PD	6172	7671	4606	2283

[1]According to Liver Cancer Study Group of Japan; [2]According to Barcelona Clinic Liver Cancer. HCV: Hepatitis C virus; HBV: Hepatitis B virus; AFP: Alfha-fetoprotein; DCP: Des-gamma-carboxy prothrombin. PD: Progressive disease; SD: Stable disease.

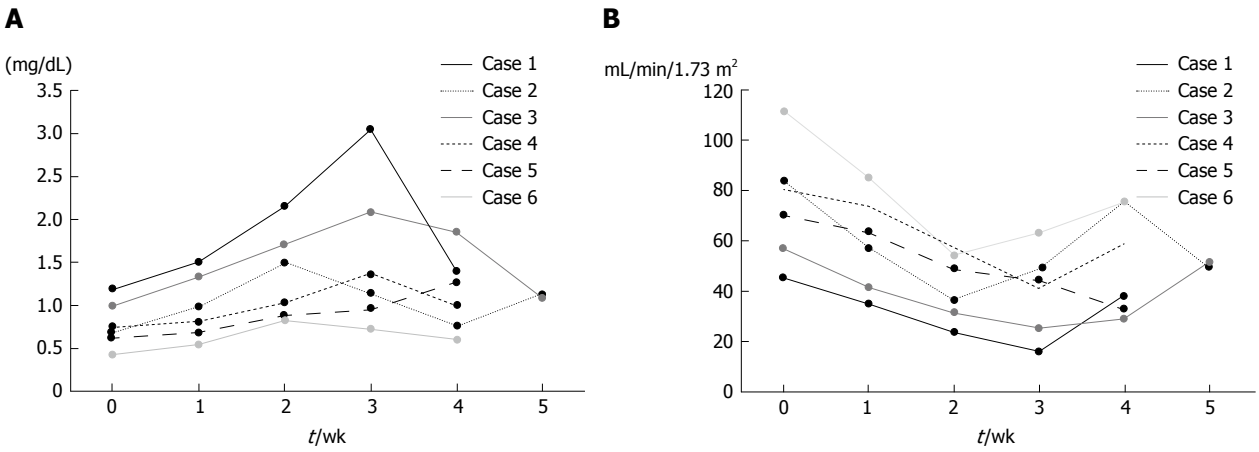

Figure 6 Changes in serum creatinine levels and eGFR of patients administered with deferasirox. A: All patients showed gradual increase in creatinine levels after initiation of DFX treatment, until reduction in DFX dose or discontinuation of treatment. Creatinine levels in all patients were improved by dose reduction. B: All patients showed gradual decrease in eGFR after initiation of DFX treatment, until reduction in DFX dose or discontinuation of treatment.

Table 3 Summary of adverse effects (mean ± SD)

Case	Creatinine at baseline (mg/dL)	eGFR at baseline (mL/min/1.73 m^2)	Adverse effects (grade)	Onset time of grade 2 adverse effects
1	1.19	45.6	Elevated creatinine (3)	Week 2
			Anorexia (2)	Week 2
2	0.70	83.7	Elevated creatinine (2)	Week 2
			Anorexia (2)	Week 3
3	1.00	56.9	Elevated creatinine (2)	Week 3
4	0.75	80.2	Elevated creatinine (2)	Week 3
5	0.63	70.2	Elevated creatinine (2)	Week 4
			Anorexia (1)	
6	0.43	111.5	Anorexia (1)	
			Elevated creatinine (1)	

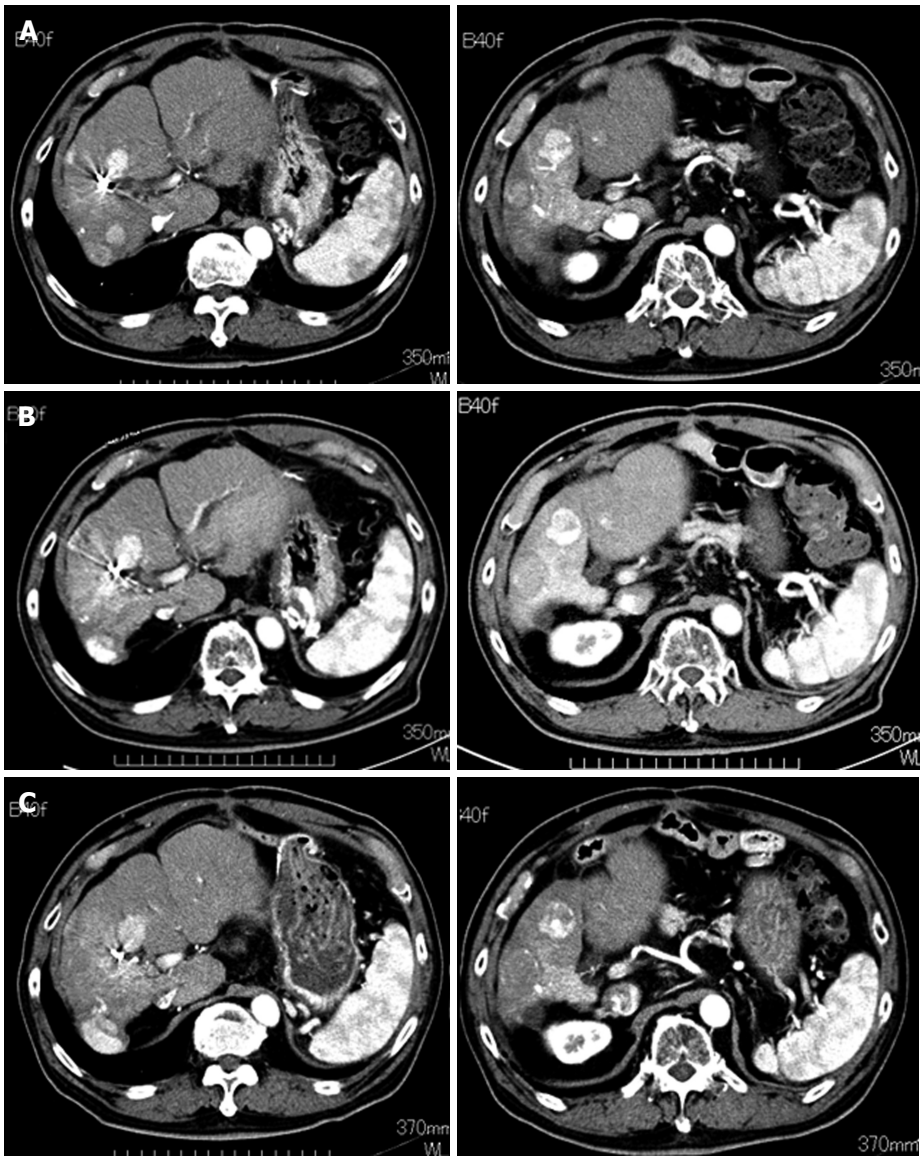

Figure 7 Progress of hepatocellular carcinoma in one patient (case 3) who maintained stable disease for eight months. The tumor size did not increase during the DFX administration period. A: Before deferasirox (DFX) administration; B: After one course of DFX therapy; C: After four courses of DFX therapy.

and one remained alive.

DISCUSSION

Sorafenib has been used worldwide as first-line chemotherapy for advanced HCC patients. However, there are currently no established second-line chemotherapies for such patients. We have been researching the efficacy of iron chelators such as DFO and DFX in basic and clinical studies[13-15,17]. We demonstrated that DFO therapy can be considered as second-line chemotherapy for advanced HCC patients refractory to chemotherapy, showing that the response to DFO therapy and the one-year survival rate were both 20%[15]. DFX is a newly developed oral iron chelator for the treatment of iron-overload disease. We demonstrated that DFX can prevent liver fibrosis and hepatocarcinogenesis in a choline-deficient L-amino acid (CDAA) diet-induced rat model of liver injury[17]. However, we have not examined the efficacy of DFX in a HCC model. Therefore, we investigate in the present study, the effect of DFX against HCC through basic and clinical research.

In the basic study, we demonstrated the inhibitory effects of DFX against HCC both *in vitro* and *in vivo*. Administration of DFX inhibited the proliferation of three hepatoma cell lines (HepG2, Hep3B, and Huh7) in a dose-dependent manner, and induced apoptosis through an increase in caspase-3 activity (Figure 2). Previous reports have shown that DFX inhibited the cell cycle at the G0/G1 and S phases, and induced apoptosis in the Huh7 human hepatoma cell line[12,24]. We showed that DFX notably inhibited the development of liver tumors and significantly upregulated the mRNA expression levels of hepcidin, transferrin receptor 1, and HIF-1α in both tumor and non-tumor areas,

compared with control (Figures 3 and 4). Iron chelators can induce hypoxia, which in turn upregulates HIF-1. Hypoxia inducible factor-1 is composed of two subunits, an α subunit that is regulated by the hypoxic state and a constitutively expressed β subunit. It also activates downstream effectors such as transferrin receptor 1, N-myc downstream regulated gene-1, and p53[9]. Subsequently, hepcidin is transcriptionally activated by p53[25]. It has been reported that hepcidin mRNA levels were significantly lower in liver tumor tissues than in the surrounding and control tissues in the DEN-induced murine hepatocarcinogenic model used in this study[26]. Furthermore, it was shown that the hepatic hepcidin expression decreased in tissues with cirrhosis and in HCC patients[27]. In our study, hepcidin mRNA levels were also reduced in tumor tissues before DFX treatment. As hepcidin is an iron homeostasis regulator, DFX can potentially improve iron homeostasis in liver tumors. Therefore, our findings suggested that DFX might inhibit tumor growth *via* hypoxia-associated factors and by regulating iron homeostasis through the upregulation of hepcidin.

Based on the results of the basic study, we proposed a clinical study of DFX therapy in advanced HCC patients refractory to chemotherapy. To our knowledge, this is the first report on DFX therapy for HCC in a clinical trial setting. To determine the recommended therapeutic dose for DFX therapy, we started at 10 mg/kg per day based on the minimal dose used in the treatment of iron-overload disease, and gradually increased the dose to a maximum of 30 mg/kg per day, if proven safe. Unfortunately, we could not continuously administer a high dose of DFX (20-30 mg/kg per day) due to AEs. Thus, the administered maintenance dose of DFX was 5-15 mg/kg per day.

Renal dysfunction, as indicated by elevated creatinine levels was observed in all six patients, while anorexia which was well tolerated, was observed in four patients (Table 3). Porter *et al*[28] reported that increased serum creatinine related to DFX was observed in 39.7% of patients with myelodysplastic syndrome and other transfusion-dependent anemias, most frequently at doses between 20 and 30 mg/kg per day; however, this increase was not progressive. Although there were no reports on the Japanese population exclusively, Kohgo *et al*[29] reported that the incidence of increased serum creatinine was 23.5% in patients from five countries, including Japan (Japanese patients, n = 53; non-Japanese patients, n = 49)[29]. In our clinical study, all six patients exhibited elevated creatinine levels (AE grade, 1-3) upon DFX administration at a dose of 20 mg/kg per day (n = 4) and 30 mg/kg per day (n = 2). However, the creatinine levels decreased after the dose was reduced (Figure 6A). In addition, DFX was associated with a higher risk of acute renal failure than DFO in a large Asian population[30]. In fact, our data showed that the incidence of increased serum creatinine was 10% (1 in 10 patients) in DFO therapy[15] and 100% in DFX therapy; however, these studies only involved small populations. A possible reason for the difference in the risk of renal dysfunction between DFX and DFO therapies is the difference in their half-lives; DFX has a relatively long half-life compared with DFO.

We demonstrated that a DFX dose of 20 mg/kg per day inhibited the development of liver tumors in a murine model. A previous report also confirmed the efficacy of DFX using the same dose in an esophageal cancer xenograft mice model[31]. However, there were no responders for DFX therapy in our clinical study; one patient exhibited SD, and four patients exhibited PD. On the other hand, it has been reported that the iron chelation activity of DFX increased in a dose-dependent manner; a decrease in serum ferritin was observed at a dose of 20 and 30 mg/kg per day, but not at 5 and 10 mg/kg per day[28]. Supplementary Table 1 shows changes in serum ferritin before and after a single course of DFX therapy. Although three patients had normal range of ferritin at baseline, there were no significant changes in serum ferritin level between baseline and one course after treatment (P = 0.12). Thus, DFX dosage of 5-15 mg/kg per day may not be adequate for not only tumor inhibition, but also for iron chelation in patients with HCC and liver cirrhosis. However, it is problematic to increase the effective dose of DFX to 20-30 mg/kg per day because of elevated creatinine levels in patients, in accordance to the treatment protocol of this clinical study; thus, a normal level of eGFR should be included as an eligibility criterion in future clinical studies. Alternatively, we previously reported that the combination therapy of DFX and sorafenib markedly inhibited liver fibrosis and hepatocarcinogenesis with a significant reduction of the AEs associated with sorafenib in a CDAA-induced rat model[17]. The efficacy of this combination therapy was also demonstrated based on *in vitro* and *in vivo* studies by other researchers[32]. If the effectiveness of DFX and sorafenib combination therapy can be verified in clinical trials, a low dose of DFX may be used as a novel HCC therapy.

In conclusion, we demonstrated the inhibitory effects of DFX against HCC in a basic study designed to investigate its antiproliferative potential. However, the efficacy of DFX in the clinical study could not be demonstrated because of inadequate doses due to DLT. Although iron chelators have promising therapeutic potential, further examinations are necessary to establish their clinical applications.

ACKNOWLEDGMENTS

We thank Mrs. Mariko Yamada, Mrs. Ihoko Fujimoto, and Mrs. Hiromi Kurose for their technical assistance and support.

COMMENTS

Background

Sorafenib is recommended as the current standard therapy for advanced hepatocellular carcinoma (HCC) patients and has been generally administered

as first-line chemotherapy for those with Child-Pugh A score. However, no established second-line chemotherapies are available for patients with Child-Pugh B score or those refractory to sorafenib. Iron is essential for a number of cellular metabolic processes including DNA synthesis. It is also required for the proliferation of cancer cells before initiation of DNA synthesis. Although iron chelators are not classified as anticancer drugs, they exert antiproliferative effects in several cancers, including HCC.

Research frontiers

The authors previously reported that deferoxamine (DFO) can prevent both liver fibrosis and development of preneoplastic lesions in rats. We also performed a pilot study of DFO in advanced HCC patients for the first time, in which we demonstrated the efficacy of this chelator. However, DFO cannot be administered orally, thus limiting its clinical application. Recently, deferasirox (DFX), a newly developed oral iron chelator, was shown to exert a potent antiproliferative effect against human hepatoma cell culture and hepatocarcinogenesis *in vivo*. We have also reported that DFX, like DFO, was able to prevent liver fibrosis and hepatocarcinogenesis in rats.

Innovations and breakthroughs

There have been no *in vivo* or clinical studies of DFX against HCC. The authors investigated for the first time, the inhibitory effects of DFX against HCC through both basic and clinical research.

Applications

The authors demonstrated the inhibitory effects of DFX against HCC in a basic study. However, the efficacy of DFX in the clinical study could not be verified owing to dose-limiting toxicity in patients. Although iron chelators have promising therapeutic potential, further examinations are necessary to establish their clinical applications.

Terminology

DFO and DFX are iron chelators that are commonly used for the treatment of iron-overload disease. DFO is ordinarily administered by intravenously, whereas DFX is a newly developed oral iron chelator.

Peer-review

The manuscript is a combination of experimental and clinical results, it is difficult to compare the experimental cell lines derived from cells of HCC. There are no substantial criticisms to the present work.

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P- Reviewer: Peng SY, Srichairatanakool S, Zielinski J
S- Editor: Qi Y **L- Editor**: A **E- Editor**: Zhang FF

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DOI: 10.3748/wjg.v22.i40.8978

World J Gastroenterol 2016 October 28; 22(40): 8978-8990
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

ORIGINAL ARTICLE

Observational Study

Factors affecting the quality of life of patients after gastrectomy as assessed using the newly developed PGSAS-45 scale: A nationwide multi-institutional study

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Supported by Jikei University School of Medicine and Japanese Society for Gastro-surgical Pathophysiology.

Institutional review board statement: This study was reviewed and approved by local ethics committees at each institution.

Clinical trial registration statement: This study was registered with the University Hospital Medical Information Network's Clinical Trials Registry (UMIN-CTR; registration number 000002116).

Informed consent statement: Written informed consent was obtained from all patients prior to study enrollment.

Conflict-of-interest statement: The authors declare no conflicts of interests related to the publication of this study.

Data sharing statement: No additional data was available.

Manuscript source: Invited manuscript

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Received: June 28, 2016
Peer-review started: June 29, 2016
First decision: July 29, 2016
Revised: August 14, 2016
Accepted: September 6, 2016
Article in press: September 6, 2016
Published online: October 28, 2016

Abstract

AIM

To identify certain clinical factors other than the type of gastrectomy which affect the postoperative quality of life (QOL) of patients after gastrectomy.

METHODS

The postgastrectomy syndrome assessment scale (PGSAS)-45 was designed to assess the severity of symptoms, the living status and the QOL of gastrectomized patients. It consists of 45 items, of which 22 are original items while 23 were retrieved from the SF-8 and Gastrointestinal Symptoms Rating Scale questionnaires with permission. A nationwide surveillance study to validate PGSAS was conducted and 2368 gastric cancer patients who underwent various types of gastrectomy at 52 medical institutions were enrolled. Of these, 1777 patients who underwent total gastrectomy (TG) reconstructed with Roux-Y (n = 393), distal gastrectomy (DG) reconstructed with Billroth-I (n = 909), or DG reconstructed with Roux-Y (n = 475) were evaluated in the current study. The influence of the type of gastrectomy and other clinical factors such as age, sex, duration after surgery, the symptom severity, the degree of weight loss, dietary intake, and the ability for working on the postoperative QOL (*i.e.*, dissatisfaction for daily life subscale, physical component summary and mental component summary of the SF-8) were examined by multiple regression analysis (MRA). In addition, importance of various symptoms such as esophageal reflux, abdominal pain, meal-related distress, indigestion, diarrhea, constipation and dumping on the postoperative living status and QOL were also appraised by MRA.

RESULTS

The postoperative QOL were significantly deteriorated in patients who underwent TG compared to those after DG. However, the extent of gastrectomy was not an influential factor on patients' QOL when adjusted by the MRA. Among various clinical factors, the symptom severity, ability for working, and necessity for additional meals were the most influential factors to the postoperative QOL. As for the individual symptoms, meal-related distress, dumping, abdominal pain, and esophageal reflux significantly affected the postoperative QOL in that order, while the influence of indigestion, diarrhea and constipation was insignificant.

CONCLUSION

Several clinical factors such as the symptom severity (especially in meal-related distress and dumping), ability for working and necessity for additional meals were the main factors which affected the patients' well-being after gastrectomy.

Key words: Postgastrectomy syndrome; Quality of life; Patient- reported outcome; Effect size; Gastrectomy

Core tip: The extent of gastrectomy has been reported to substantially affect the postoperative quality of life (QOL). However, considerable differences in the QOL have been observed among patients who underwent the same type of gastrectomy, implicating that other clinical factors may have major influence over the postoperative QOL. In the present study, we first found that several clinical factors such as the symptom severity, ability for working and necessity for additional meals had significant impact on the postoperative QOL, while the influence of the extent of gastrectomy was unexpectedly small. These findings give us deeper understanding to manage the postgastrectomy syndrome appropriately.

Nakada K, Takahashi M, Ikeda M, Kinami S, Yoshida M, Uenosono Y, Kawashima Y, Nakao S, Oshio A, Suzukamo Y, Terashima M, Kodera Y. Factors affecting the quality of life of patients after gastrectomy as assessed using the newly developed PGSAS-45 scale: A nationwide multi-institutional study. *World J Gastroenterol* 2016; 22(40): 8978-8990 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i40/8978.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i40.8978

INTRODUCTION

Gastrectomy is widely used as an effective curative treatment modality in patients with gastric cancer. In Japan, the rate of diagnosis of gastric cancer at an early stage has been increasing[1], and with the consequent improvement of the treatment results, greater attention is being paid to the postoperative quality of life (QOL) of patients who underwent gastrectomy. Various clinical problems may occur after gastrectomy, including various abdominal and systemic symptoms, restriction of food intake, weight loss, decrease in physical activity, *etc.*, which can interfere with the QOL of gastrectomized patients. Thus, there is need to prevent and manage these sequelae after

gastrectomy, collectively labeled as postgastrectomy syndrome (PGS)[2-7].

Until date, the influence of the type of gastrectomy on the risk of development of PGS has been mainly investigated[8-23]. Multiple studies have reported a greater deterioration of the QOL after total gastrectomy (TG) than distal gastrectomy (DG)[8,10,13,18,19], and it is thought that the more extensive the resection of the stomach, the greater the severity of PGS[11]. On the other hand, function-preserving gastrectomy, in which the extent of gastrectomy is reduced, such as pylorus-preserving gastrectomy (PPG)[9,11,16,17,23] and proximal gastrectomy[20], is often used for treating early gastric cancer and has been reported to be useful to improve the QOL of patients after surgery. Thus, improvement in the gastrectomy procedures is recognized as one of the reliable means to reduce the risk of development of PGS. However, at least at present, it is difficult to eliminate PGS completely only by improving the gastrectomy procedures.

It has been observed that there are considerable individual differences in the postoperative QOL among patients who underwent the same type of gastrectomy; therefore, it appears likely that clinical factors other than the type of gastrectomy may also significantly influence the postoperative QOL, although not much information on this is available yet. Therefore, it seems important to identify undiscovered clinical factors which might affect the postoperative QOL of patients who underwent gastrectomy, besides the type of gastrectomy performed, in order to obtain a deeper understanding of PGS and to develop effective methods of prevention and management. Various symptoms are known to develop after gastrectomy, which cause much discomfort to the patients and place a burden on their lives. Although the degree of influence of these symptoms on the patients' daily lives and QOL appears to differ depending on the nature of symptoms, the differences in the influences of each symptom on the patients' daily lives and QOL have not yet been clarified. Therefore, this study was conducted to clarify these issues in the patients who underwent gastrectomy.

MATERIALS AND METHODS

Patients

Fifty-two institutions participated in this study. Patient eligibility criteria were: (1) diagnosis of pathologically-confirmed stage IA or IB gastric cancer; (2) first-time gastrectomy status; (3) age $\geqslant$ 20 and $\leqslant$ 75 years; (4) no history of chemotherapy; (5) no recurrence or distant metastasis indicated; (6) gastrectomy conducted one or more years prior to enrollment date; (7) performance status $\leqslant$ 1 on the Eastern Cooperative Oncology Group scale; (8) full capacity to understand and respond to the questionnaire; (9) no history of other diseases or surgeries which might influence responses to the questionnaire; (10) no presence of organ failure or mental illness; and (11) written informed consent. Patients with dual malignancy or concomitant resection of other organs (with co-resection equivalent to cholecystectomy being the exception) were excluded.

QOL assessment

The Postgastrectomy Syndrome Assessment Scale (PGSAS)-45[24] is a newly developed, multidimensional quality of life questionnaire (QLQ) based on the 8-item short-form health survey (SF-8)[25] and the Gastrointestinal Symptom Rating Scale (GSRS)[26]. The PGSAS-45 questionnaire consists of a total of 45 questions (Table 1), with eight items from the SF-8, 15 items from the GSRS, and 22 clinically-important items selected by the Japan Postgastrectomy Syndrome Working Party (JPGSWP) (Table 2). The PGSAS-45 questionnaire (Table 1) includes 23 items pertaining to postoperative symptoms (items 9-33), including 15 items from the GSRS and 8 newly selected items. In addition, 12 questionnaire items pertaining to dietary intake (8 items), work (1 item), and level of satisfaction with daily life (3 items) were selected. Twenty-three symptom items were clustered into seven symptom subscales (SS), *i.e.,* the esophageal reflux SS, abdominal pain SS, meal-related distress SS, indigestion SS, diarrhea SS, constipation SS, and dumping SS by factor analysis. Details of the PGSAS-45 have been reported previously[24].

Study methods

This study utilized continuous sampling from a central registration system for participant enrollment. The questionnaire was distributed to all eligible patients as they presented to participating clinics. After completing the questionnaire, patients were instructed to return forms to the data center. All QOL data from questionnaires were matched with individual patient data collected *via* case report forms. This study was registered with the University Hospital Medical Information Network's Clinical Trials Registry (UMIN-CTR; registration number 000002116). This study was approved by local ethics committees at each institution. Written informed consent was obtained from all enrolled patients. Of the 2922 patients who were handed the questionnaire sheets between July 2009 and December 2010, 2520 (86%) responded and 2368 were confirmed to be eligible for the study (Figure 1). Of these, data from 1777 patients who underwent either TG or DG were used in the current study.

Statistical analysis

In comparing patients' characteristics, living status and QOLs after TG and DG, statistical methods included the *t* test and χ^2 test. The effects of various clinical factors such as type of gastrectomy as well as age, sex, postoperative period, the severity of symptoms, the degree of body weight loss, the necessity for additional

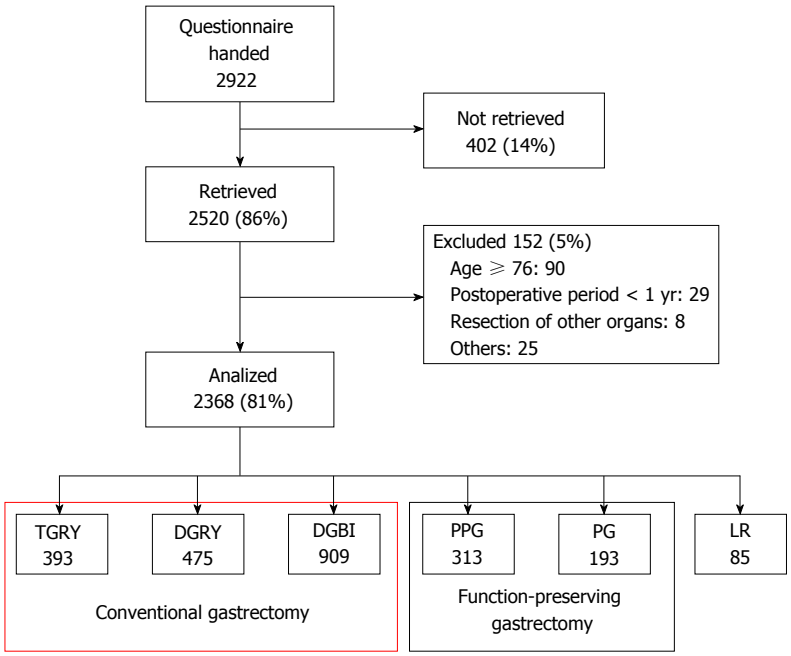

Figure 1 Outline of the Study. TGRY: Total gastrectomy with Roux-en-Y reconstruction; DGRY: Distal gastrectomy with Roux-en-Y reconstruction; DGBI: Distal gastrectomy with Billroth I reconstruction; PPG: Pylorus preserving gastrectomy; PG: Proximal gastrectomy; LR: Local resection.

food and the ability for working on the patients' QOL were investigated by multiple regression analysis (MRA). Moreover, the impact of seven symptom SS on the living status and QOL of patients after gastrectomy were examined by MRA. The values of $P < 0.05$ were considered significant. To evaluate effect sizes, Cohen's *d*, standardization coefficient of regression (β) and coefficient of determination (R^2) were used. Interpretation of effect sizes were ⩾ 0.2 small, ⩾ 0.5 medium, and ⩾ 0.8 large in Cohen's *d*; ⩾ 0.1 small, ⩾ 0.3 medium, and ⩾ 0.5 large in β; ⩾ 0.02 small, ⩾ 0.13 medium, and ⩾ 0.26 large in R^2. Statistical analyses were performed by the biostatisticians mainly using StatView for Windows Ver. 5.0 (SAS Institute Inc.).

RESULTS

Comparison of patients' characteristics, living status and QOL between TG and DG

Of the 1777 patients treated by gastrectomy who were included in this study, 393 underwent TG and 1384 underwent DG (B-I method in 909 patients and Roux-en-Y method in 475 patients). Comparison between the patients who underwent TG (TG group) and those who underwent DG (DG group) revealed that while the mean "age" was significantly higher in the TG group, there were no significant differences in the "sex" distribution and "postoperative period" between the two groups. "Symptoms", three evaluation items; "change in body weight", "necessity for additional meals", and "ability for working"; which represent the living status, and another three evaluation items; "dissatisfaction for daily life SS", "physical component summary (PCS)", and "mental component summary (MCS)"; which represent the QOL were significantly worse in the TG group than in the DG group. Calculation of Cohen's *d* effect sizes indicated that there were moderate differences in the influences of "change in body weight", "necessity for additional meals" and "dissatisfaction for daily life SS", and slight differences in the influence of "symptoms" and "ability for working" between the TG and DG groups. On the other hand, although there were statistically significant differences in the influence of "PCS" and "MCS" between the two groups, the Cohen's *d* effect sizes were very small (< 0.2), indicating the absence of any clinically meaningful differences (Table 3).

Influence of the type of gastrectomy and various other clinical factors on the postoperative QOL of gastrectomy patients

MRA using the type of gastrectomy, patient's characteristics, symptom and living status as predictor variables was performed to assess the influence of each factor on the three integrated outcome measures for the QOL domain. "Symptoms" and "ability for working" significantly affected on all the QOL outcome measures, with medium effect sizes (β ⩾ 0.3). In addition, "necessity for additional meals" significantly affected on the "dissatisfaction for daily life SS" with a small effect size (β ⩾ 0.1). "Age" marginally affected on the "dissatisfaction for daily life SS" and "MCS" with $\beta = 0.09$. On the other hand, "type of gastrectomy", "sex", "postoperative period" and "change in body weight" had an effect size of $\beta < 0.09$ on all the QOL outcome measures, while some clinical factors had a

Table 1 Structure of postgastrectomy syndrome assessment scale-45

Domains	Subdomains		Items		Subscales
QOL	SF-8 (QOL)	1	Physical functioning[1]	Five or six-point Likert scale	Physical component summary[1]
		2	Role physical[1]		Mental component summary[1]
		3	Bodily pain[1]		
		4	General health[1]		
		5	Vitality[1]		
		6	Social functioning[1]		
		7	Role emotional[1]		
		8	Mental health[1]		
Symptoms	GSRS symptoms	9	Abdominal pains	Seven-point Likert scale Except item 29 and 32	Esophageal reflux subscale (item 10, 11, 13, 24)
		10	Heartburn		Abdominal pain subscale (item 9, 12, 28)
		11	Acid regurgitation		Meal-related distress subscale (item 25-27)
		12	Sucking sensations in the epigastrium		Indigestion subscale (item 14-17)
		13	Nausea and vomiting		Diarrhea subscale (item 19, 20, 22)
		14	Borborygmus		Constipation subscale (item 18, 21, 23)
		15	Abdominal distension		Dumping subscale (item 30, 31, 33)
		16	Nausea and vomiting		
		17	Increased flatus		Total symptom scale (above seven subscales)
		18	Decreased passage of stools		
		19	Increased passage of stools		
		20	Loose stools		
		21	Hard stools		
		22	Urgent need for defecation		
		23	Feeling of incomplete evacuation		
	PGSAS original symptoms	24	Bile regurgitation		
		25	Sense of foods sticking		
		26	Postprandial fullness		
		27	Early satiation		
		28	Lower abdominal pains		
		29	Number and type of early dumping symptoms		
		30	Early dumping general symptoms		
		31	Early dumping abdominal symptoms		
		32	Number and type of late dumping symptoms		
		33	Late dumping symptoms		
Living status	Meals (amount) 1	34	Ingested amount of food per meal[1]		-
		35	Ingested amount of food per day[1]		
		36	Frequency of main meals		
		37	Frequency of additional meals		
	Meals (quality)	38	Appetite[1]	Five-point Likert scale	Quality of ingestion subscale[1] (item 38-40)
		39	Hunger feeling[1]		
		40	Satiety feeling[1]		
	Meals (amount) 2	41	Necessity for additional meals	Five-point Likert scale	-
	Social activity	42	Ability for working	Five-point Likert scale	-
QOL	Dissatisfaction (QOL)	43	Dissatisfaction with symptoms	Five-point Likert scale	Dissatisfaction for daily life subscale (item 43-45)
		44	Dissatisfaction at the meal		
		45	Dissatisfaction at working		

In items or subscales without[1], higher score indicating worse condition; in items or subscales with[1], higher score indicating better condition. Each subscale is calculated as the mean of composed items or subscales, except physical component summary and mental component summary of SF-8. Item 29 and 32 don't have score. Then, they were analyzed separately.

statistically significant, but no clinically meaningful, influence. In addition, R^2, which represents the degree of influence of all the predictor variables used in the analysis, was the greatest for the "dissatisfaction for daily life SS" (R^2 = 0.606), followed by "PCS" (R^2 = 0.368) and "MCS" (R^2 = 0.333), with large effect sizes and significant influences on all the QOL outcome measures (Table 4).

Influence of various clinical factors on the postoperative QOL of gastrectomy patients; subgroup analysis by the type of gastrectomy

To clarify in greater detail the clinical factors, other than the type of gastrectomy which was identified as a significant factor as shown above, that may affect the postoperative QOL of gastrectomy patients, subgroup analysis was conducted for each type of gastrectomy.

Table 2 Postgastrectomy syndrome assessment scale original items English version

PGSAS-45 is consisting of SF-8 (item 1-8), GSRS (item 9-23) and PGSAS original items (item 24-45)	
24	Have you been bothered by bile regurgitation (having a bitter taste in your mouth) during the past month?
25	Have you been bothered by sense of foods sticking when swallowing during the past month? (Sticking food refers to uncomfortable feeling with foods piled up in the chest.)
26	Have you been bothered by postprandial fullness during the past month? (Fullness refers to uncomfortable or heavy feeling with foods piled up in the stomach.)
27	Have you been bothered by being unable to eat enough because you feel full before you finish your meal during the past month?
28	Have you been bothered by circumumbilical pains or lower abdominal pains during the past month?
29	Have you experienced following symptoms around 30 min after eating during the past month? Please encircle the number that describes your symptom. (Please check all the symptoms you have experienced.) (Ans. Q29) 1. No symptoms below [You have experienced following general symptoms.] 2. Cold sweat 3. Palpitations 4. Dizziness 5. Numbness 6. Fainting 7. Facial flushing 8. Facial pallor 9. Feeling hot 10. Fatigue or weakness 11. Lassitude 12. Drowsiness 13. Headache 14. Heaviness of the head 15. Tightness in the chest [You have experienced following abdominal symptoms.] 16. Borborygmi (except after drinking milk) 17. Abdominal cramps (except after drinking milk) 18. Diarrhoea (except after drinking milk) 19. Nausea 20. Vomiting 21. Bloating 22. Abdominal discomfort
30	For those who encircled any of the general symptom-related items in Question 29, to what extent have you been bothered by all these general symptoms during the past month?
31	For those who encircled any of the abdominal symptom-related items in Question 29, to what extent have you been bothered by all these abdominal symptoms during the past month?
32	Have you experienced following symptoms within two to three hours after eating during the past month? Please circle the number that describes your symptom. (Please check all the symptoms you have experienced.) (Ans. Q32) 1. No symptoms below [You have experienced following general symptoms.] 2. Cold sweat 3. Palpitations 4. Dizziness 5. Headache 6. Fainting 7. Fatigue or weakness 8. Lassitude 9. Languor 10. Shakiness 11. Hunger 12. Shortness of breath
33	For those who encircled any of the general symptom-related items in Question 32, to what extent have you been bothered by all these general symptoms during the past month? (Ans. Q24-28, 30 ,31, 33) 1. No discomfort at all 2. Slight discomfort 3. Mild discomfort 4. Moderate discomfort 5. Moderately severe discomfort 6. Severe discomfort 7. Very severe discomfort
34	On average what percent of preoperative food intake have you taken in single meal during the past month? (Ans. Q34) About ( ) % of the preoperative single ingested amount
35	On average, what percent of preoperative food intake have you taken per day during the past month? (Ans. Q35) About ( ) % of the preoperative total daily ingested amount
36	On average, how many main meals have you taken per day during the past month? (Ans. Q36) About ( ) times per day
37	On average, how often have you taken additional meals (light meal or snack) per day during the past month? (Ans. Q37) About ( ) times per day
38	Have you had appetite during the past month?
39	Have you felt hunger during the past month?
40	Have you felt satiety during the past month? (Satiety refers to comfortable feeling with your stomach being full.) (Ans. Q38-40) 1. Never 2. Occasionally (less than once a week) 3. Often (twice to three times per week) 4. Frequently (four to six times per week) 5. Always (every day)
41	Please encircle the number that most accurately describes the necessity for additional meals (light meal or snack) during the past month? (Ans. Q41) 1. Food intake was enough with main meals; three times per day. 2. Food intake was slightly insufficient with main meals; three times per day, and you sometimes needed to take additional meals. 3. Food intake was significantly insufficient with main meals; three times per day, and you had to take additional meals. 4. Even though you had taken additional meals besides main meals; three times per day, food intake was insufficient. 5. Food intake was insufficient because you were not able to take additional meals besides breakfast, lunch and dinner.
42	Please encircle the number which exactly describes your living status (ability for working or housekeeping) during the past month? (Ans. Q42) 1. You were able to handle your work or housework sufficiently and could even manage to work overtime. You enjoyed trip, sports, leisure activities, and dining out as you used to before operation. 2. You were able to work or handle housework as usual (By work as usual we mean during normal working hours without overtime). (You felt no difficulty when avoiding excessive work) 3. You had some difficulties with working or keeping house. You were able to handle lighter duties (70 to 80 percent of the previous activities).

4. You had moderate difficulties with working or keeping house (about 50 percent of the previous activities).
5. You could scarcely work or keep house.

43 How often have you felt dissatisfied with the chest or abdominal symptoms due to gastrectomy during the past month?

44 How often have you felt dissatisfied with being unable to eat as intended due to gastrectomy during the past month? ("being unable to eat as intended" here means that you are not able to eat what you like, with no limitation in amount and in speed.)

45 How often have you felt dissatisfied with your limited daily activities (working or housekeeping) due to gastrectomy during the past month? (Ans. Q43-45)

1. Not at all 2. Slightly 3. Moderately 4. Significantly 5. Extremely

PGSAS-45 original items [item 24-45] English version 1.0 © 2016 K Nakada, M Takahashi

Table 3 Comparison of patients' characteristics, living status and quality of life between total and distal gastrectomy

		TG (n = 393)		DG (n = 1384)		P value	Cohen's d
		mean	SD	mean	SD		
Patients' characteristics	Age (yr)	63.4	9.2	61.8	9.1	0.0019[2]	(0.18)
	Sex (male: n/%)	276/71.0%		912/66.2%		0.0798[3]	-
	Postoperative period (mo)	35.0	24.6	37.9	27.4	0.0918[2]	(0.10)
Symptoms	Total symptom score	2.2	0.7	1.9	0.7	< 0.0001[2]	0.35
Living status	Change in body weight (%)[1]	-13.8%	7.9%	-8.3%	7.6%	< 0.0001[2]	0.71
	Necessity for additional meals	2.4	0.8	1.9	0.8	< 0.0001[2]	0.61
	Ability for working	2.0	0.9	1.8	0.9	< 0.0001[2]	0.31
QOL	Dissatisfaction for daily life SS	2.3	0.9	1.9	0.8	< 0.0001[2]	0.53
	Physical component summary[1]	49.6	5.6	50.6	5.6	0.0020[2]	(0.18)
	Mental component summary[1]	49.2	6.0	49.9	5.7	0.0426[2]	(0.12)
The interpretation of effect size		Cohen's d					
None-very small		< (0.2)					
Small		⩾ 0.2					
Medium		⩾ 0.5					
Large		⩾ 0.8					

Outcome measures with[1], higher score indicating better condition; Outcome measures without[1], higher score indicating worse condition. [2]t-test; [3]χ^2 test. TG: Total gastrectomy; DG: Distal gastrectomy; QOL: Quality of life.

Table 4 Clinical factors affecting quality of life in the patients after gastrectomy (Multiple Regression Analysis)

	Dissatisfaction for daily life SS		Physical component summary[1]		Mental component summary[1]	
	β	P value	β	P value	β	P value
Type of gastrectomy [TG]	(0.047)	0.0132	(0.008)	NS	(-0.056)	0.0238
Age	(-0.091)	< 0.0001	(-0.052)	0.0236	(0.090)	0.0002
Sex [Male]	(-0.016)	NS	(0.043)	0.0576	(0.025)	NS
Period after gastrectomy	(-0.026)	NS	(-0.019)	NS	(-0.004)	NS
Total symptoms score	0.429	< 0.0001	-0.354	< 0.0001	-0.357	< 0.0001
Change in body weight[1]	(-0.036)	0.0551	(0.026)	NS	(-0.008)	NS
Necessity for additional meals	0.176	< 0.0001	(0.057)	0.0206	(-0.020)	NS
Ability for working	0.360	< 0.0001	-0.377	< 0.0001	-0.321	< 0.0001
R^2 (P value)	0.606	< 0.0001	0.368	< 0.0001	0.333	< 0.0001
If β is positive, the score of the outcome measure of the patients belonging to the category in [brackets] is higher in cases when the factor is a nominal scale, and the score of outcome measure of the patients with larger values is higher in cases when the factor is a numeral scale.						
The interpretation of effect size	β	R^2				
None-very small	< (0.100)	< (0.020)				
Small	⩾ 0.100	⩾ 0.020				
Medium	⩾ 0.300	⩾ 0.130				
Large	⩾ 0.500	⩾ 0.260				

Outcome measures with[1], higher score indicating better condition; outcome measures without[1], higher score indicating worse condition. TG: Total gastrectomy.

Like in the analysis for the type of gastrectomy, overall, "symptoms" and "ability for working" were found to have a significant influence on all the QOL outcome measures with medium effect sizes (β ⩾ 0.3) in both the TG and DG groups (although only the effect size on the "MCS" in the DG group was β = 0.289). In addition, "necessity for additional meals" significantly affected on the "dissatisfaction for daily life SS" with a small effect size (β ⩾ 0.1) in both the TG and DG groups. "Age" also significantly affected on all the QOL outcome measures with small effect sizes (β ⩾ 0.1) in the TG group. However, in the DG group, "age" was not significant, or had very small effect sizes even in case it was significant, suggesting

Table 5 Clinical factors affecting quality of life in the patients after each of total or distal gastrectomy (Subgroup Multiple Regression Analysis)

	Dissatisfaction for daily life SS		Physical component summary[1]		Mental component summary[1]	
	β	*P* value	β	*P* value	β	*P* value
TG						
Age	-0.135	0.0007	-0.160	0.0006	0.118	0.0141
Sex (male)	(0.003)	NS	(0.065)	NS	(-0.074)	NS
Period after gastrectomy	(-0.036)	NS	(0.029)	NS	(-0.054)	NS
Total symptoms score	0.428	< 0.0001	-0.441	< 0.0001	-0.350	< 0.0001
Change in body weight[1]	(-0.001)	NS	(0.020)	NS	(-0.034)	NS
Necessity for additional meals	0.281	< 0.0001	(0.085)	0.0653	(-0.028)	NS
Ability for working	0.335	< 0.0001	-0.334	< 0.0001	-0.415	< 0.0001
R^2 (*P* value)	0.565	< 0.0001	0.434	< 0.0001	0.393	< 0.0001
DG						
Age	(-0.081)	0.0001	(-0.018)	NS	(0.081)	0.0029
Sex (male)	(0.025)	NS	(0.036)	NS	(0.052)	0.0502
Period after gastrectomy	(-0.027)	NS	(-0.033)	NS	(0.011)	NS
Total symptoms score	0.441	< 0.0001	-0.316	< 0.0001	-0.355	< 0.0001
Change in body weight[1]	(-0.044)	0.0265	(0.024)	NS	(0.006)	NS
Necessity for additional meals	0.139	< 0.0001	(0.043)	NS	(-0.019)	NS
Ability for working	0.377	< 0.0001	-0.390	< 0.0001	-0.289	< 0.0001
R^2 (*P* value)	0.598	< 0.0001	0.347	< 0.0001	0.322	< 0.0001

If β is positive, the score of the outcome measure of the patients belonging to the category in [brackets] is higher in cases when the factor is a nominal scale, and the score of outcome measure of the patients with larger values is higher in cases when the factor is a numeral scale.

The interpretation of effect size	β	R^2
None-very small	< (0.100)	< (0.020)
Small	⩾ 0.100	⩾ 0.020
Medium	⩾ 0.300	⩾ 0.130
Large	⩾ 0.500	⩾ 0.260

Outcome measures with[1], higher score indicating better condition; outcome measures without[1], higher score indicating worse condition. TG: Total gastrectomy; DG: Distal gastrectomy.

that "age" had any clinically meaningful influence. "Sex", "postoperative period" and "change in body weight" had an effect size of β < 0.09 on all the QOL outcome measures in both groups, and while the influence was statistically significant in some cases, it was not clinically meaningful. Like in the analysis for the type of gastrectomy, overall, R^2 was greatest for the "dissatisfaction for daily life SS", followed by that for the "PCS" and "MCS", with large effect sizes and significant influences on all the integrated QOL outcome measures (Table 5).

Influence of the seven symptom SS on the living status and postoperative QOL of gastrectomy patients

The influence of the seven symptoms SS often found after gastrectomy, *i.e.,* "esophageal reflux", "abdominal pain", "meal-related distress", "indigestion", "diarrhea", "constipation" and "dumping", on the living status and integrated outcome measures for the QOL domain in gastrectomized patients was assessed by MRA. The results revealed that the influence on the living status and QOL outcome measures greatly differed depending on the nature of symptoms. "Meal-related distress" and "dumping" significantly affected almost all the QOL outcome measures with small effect sizes (β ⩾ 0.1). In addition, "abdominal pain" and "esophageal reflux" significantly affected some of the outcome measures ("PCS" and "dissatisfaction for daily life SS") with small effect sizes (β ⩾ 0.1). On the other hand, "indigestion", "diarrhea" and "constipation" had no clinically meaningful influence on any of the QOL outcome measures, with β < 0.09. The R^2 was the greatest for "dissatisfaction for daily life SS" and "PCS" (significant influence with large effect sizes, R^2 ⩾ 0.26), followed by "MCS", "ability for working" and "necessity for additional meals" (significant influence with medium effect sizes, R^2 ⩾ 0.13) and "change in body weight" (significant influence with a small effect size, R^2 ⩾ 0.02) (Table 6).

DISCUSSION

Multiple studies have reported the influence of different gastrectomy procedures on the postoperative QOL of the surgically treated patients[8-23], however, the influences of other clinical factors on the postoperative QOL are still unknown. The present study was conducted to clarify the influences of various clinical factors on the QOL of patients after gastrectomy using PGSAS-45; a newly developed composite questionnaire for postgastrectomy evaluation. The results of our evaluation revealed that among a variety of clinical factors, "symptoms" had the strongest influence on the postoperative QOL of gastrectomy patients, followed by "ability for working" and "necessity for additional meals". In addition, among the symptoms, "meal-related distress" and "dumping" affected the postoperative QOL the most strongly and broadly, and

Table 6 Influence of various symptoms on patients' living status and quality of life (Multiple regression analysis)

	Change in body weight[1]		Necessity for additional meals		Ability for working		Dissatisfaction for daily life SS		Physical component summary[1]		Mental component summary[1]	
	β	*P* value	β	*P* value	β	*P* value	β	*P* value	β	*P* value	β	*P* value
Esophageal reflux SS	(-0.04)	NS	(0.052)	NS	(0.081)	0.0126	(0.085)	0.0011	-0.126	< 0.0001	(-0.085)	0.0062
Abdominal pain SS	(0.042)	NS	(-0.004)	NS	(0.096)	0.0046	0.146	< 0.0001	-0.261	< 0.0001	(-0.094)	0.0039
Meal-related distress SS	-0.170	< 0.0001	0.279	< 0.0001	0.116	0.0012	0.282	< 0.0001	(-0.074)	0.0263	-0.144	< 0.0001
Indigestion SS	(-0.036)	NS	(0.004)	NS	(-0.001)	NS	(0.015)	NS	(0.024)	NS	(-0.058)	0.0699
Diarrhea SS	(0.023)	NS	(-0.037)	NS	(-0.076)	0.0061	(0.011)	NS	(0.022)	NS	(-0.054)	0.0441
Constipation SS	(0.062)	0.0431	(0.007)	NS	(0.093)	0.0008	(0.006)	NS	(-0.037)	NS	(-0.056)	0.0356
Dumping SS	(-0.051)	NS	0.113	0.0015	0.214	< 0.0001	0.283	< 0.0001	-0.168	< 0.0001	-0.141	< 0.0001
R^2 (*P* value)	0.040	< 0.0001	0.148	< 0.0001	0.202	< 0.0001	0.483	< 0.0001	0.276	< 0.0001	0.240	< 0.0001
If β is positive, the score of outcome measure of the patients with larger values is higher												
The interpretation of effect size		β	R^2									
None-very small		< (0.100)	< (0.020)									
Small		⩾ 0.100	⩾ 0.020									
Medium		⩾ 0.300	⩾ 0.130									
Large		⩾ 0.500	⩾ 0.260									

[1]Outcome measures with, higher score indicating better condition; outcome measures without, higher score indicating worse condition.

"abdominal pain" and "esophageal reflux" also affected some of the outcome measures, but to a limited extent. "Indigestion", "diarrhea" and "constipation" had the smallest influence. "Change in body weight", which is often used as an objective evaluation index after gastrectomy, had no clinically meaningful influence on the living status or the QOL of postgastrectomy patients. This is the first study to investigate the influence of a variety of clinical factors other than type of gastrectomy on the postoperative QOL of gastrectomy patients.

PGS occurs frequently after gastrectomy that may cause significant clinical problems[2-7]. Clinical features of PGS include the occurrence of various symptoms, reduced dietary intake, weight loss, reduced physical activity, and reduced physical and mental QOL. PGS is usually the most severe after TG, and the postoperative QOL of patients is known to be better after DG, in which the proximal stomach is partially preserved, than after TG[8,10,13,18,19]. Our study also showed a significantly higher "necessity for additional meals", greater "weight loss", lower "ability for working" and worse "dissatisfaction for daily life SS", "PCS" and "MCS" of SF-8 in the TG group than in the DG group. Furthermore, it has been reported that function-preserving gastrectomy, such as PPG and proximal gastrectomy, is associated with a better postoperative QOL as compared to gastrectomy[9,11,16,17,20,23]. Thus, the type of gastrectomy is a well-known factor affecting the postoperative QOL of patients who underwent gastrectomy, and improvement in the gastrectomy procedures may be expected to improve the postoperative QOL of gastrectomy patients. Accordingly, it is clinically important to make efforts to further improve the surgical procedures of gastrectomy. On the other hand, at present, we know that improvement in the gastrectomy procedures can only reduce, though not completely eliminate the development of PGS, and that its effect is limited. Therefore, it is necessary to identify other clinical factors that may affect the postoperative QOL of gastrectomy patients besides the type of gastrectomy, and to manage PGS in a multifaceted manner for further improvement of the postoperative QOL of gastrectomy patients.

It is well-known that there are considerable individual differences in the degree of interference with the daily life activities among patients who underwent the same type of gastrectomy, suggesting that clinical factors other than the type of gastrectomy also have a significant influence on the postoperative QOL of gastrectomy patients, although there is little information yet on such factors. In the present study, we investigated the influence of clinical factors such as "age", "sex", "postoperative period", "symptoms", "change in body weight", "necessity for additional meals" and "ability for working", on the postoperative QOL of gastrectomy patients by conducting MRA of the data of 1777 patients who underwent gastrectomy. According to the obtained results, among these clinical factors, "symptoms", "the ability for working" and "the necessity for additional meals" had a significant influence on the integrated QOL outcome measures; "symptoms" had the strongest influence,

followed by "ability for working" and "necessity for additional meals". In particular, "symptoms" and "ability for working" significantly affected all the integrated QOL outcome measures with considerable effect sizes, suggesting that they can be counted as reliable factors adversely affecting the postoperative QOL of gastrectomy patients.

"Change in body weight" is often used as an index which objectively evaluates the physical status of postgastrectomy patients[21,22,27,28], but in this study, the influence of "change in body weight" per se on the postoperative QOL of gastrectomy patients was unexpectedly small.

In regard to the influence of "type of gastrectomy" performed, univariate analysis (Table 3) revealed that many outcome measures for living status and QOL were significantly worse in the TG group than in the DG group, with moderate to small effect sizes, indicating considerable differences in the effects among the type of gastrectomy. However, the multivariate analysis (conducted by us) revealed that the influence of "type of gastrectomy" per se on "dissatisfaction for daily life SS", "PCS" and "MCS" was very small, based on the effect sizes. These results suggest that the differences in the living status and QOL between the TG and DG groups were caused not by the direct influence of the gastrectomy procedures, but rather, by the indirect influence to the QOL that might affect the factors such as "symptoms", "necessity for additional meals", and "ability for working". Other clinical factors such as "age", "sex" and "postoperative period" not shorter than 1 year, had little, if any, or no influence on the postoperative QOL in gastrectomy patients.

The results of subgroup analysis of the influence of these clinical factors on the postoperative QOL for each type of gastrectomy using MRA were similar to those of the analysis for all type of gastrectomy, suggesting that multiple clinical factors other than the type of gastrectomy, *i.e.,* "symptoms", "necessity for additional meals" and "ability for working", have a definite influence on the QOL of postgastrectomy patients.

Identification of clinical factors affecting the QOL of gastrectomized patients and obtaining a deeper understanding of PGS is expected to be useful for the better management of PGS, besides providing clues to improve the gastrectomy procedures. Individual differences in the adaptability to gastrointestinal dysfunction caused by gastrectomy, patient food preferences, how to eat meals (*e.g.,* overeating or eating quickly), *etc.,* are also expected to affect these clinical factors, and it is desirable to support the reconstruction of the dietary habits according to the adaptability of each patient after gastrectomy.

MRA showed that the R^2 was 0.606 for "dissatisfaction for daily life SS", 0.368 for SF-8 "PCS" and 0.333 for SF-8 "MCS"; thus, among the integrated QOL outcome measures, R^2 for "dissatisfaction for daily life SS" was exceptionally high. This indicates that "dissatisfaction for daily life SS" most appropriately reflects the influence of all the predictive variables used in the analysis, and that "dissatisfaction for daily life SS" is a valid comprehensive index to evaluate the postoperative QOL after gastrectomy.

The present study revealed that among a variety of clinical factors, "symptoms" had the greatest influence on the QOL after gastrectomy. Comparison in greater detail of the influences of the symptom SS on the living status and QOL outcome measures by MRA revealed that "dumping" and "meal-related distress" had significant and the strongest influence on almost all of the main outcome measures for the living status and QOL domains. "Abdominal pain" and "esophageal reflux" affected some of the outcome measures ("PCS" and "dissatisfaction for daily life SS"). On the other hand, "indigestion", "diarrhea" and "constipation" scarcely affected the activities of daily life or the QOL. Thus, our results revealed that the influence on the daily life activities and QOL of postgastrectomy patients differed significantly among the various symptoms. Though it is well known that a variety of symptoms occurring after gastrectomy decrease the QOL of postoperative patients, this is the first study that weighed the size of influence by the nature of symptoms.

"Dumping" and "meal-related distress" are characteristic symptoms frequently found after gastrectomy, and are well-known as dumping syndrome and small stomach syndrome, respectively[3,29,30]. The present study revealed that these symptoms are clinically extremely important, because they have the greatest effect of interfering with the daily life activities and reducing the postoperative QOL of gastrectomy patients. Thus, improvement of the gastrectomy procedures to reduce these symptoms will contribute to the improvement of the postoperative QOL of gastrectomy patients. Namely, gastrectomy procedures that preserve the pylorus and prevent dumping (such as PPG and proximal gastrectomy) may be expected to reduce dumping symptoms and those that increase the residual gastric volume (such as a reduced extent of gastrectomy and creation of a substitute stomach) may be expected to reduce small stomach symptoms, which would be clinically useful. Therefore, it would be desirable to further improve the gastrectomy procedures with the objective of reducing these symptoms and to evaluate their efficacy using appropriate patient-reported outcome measures.

So far, various questionnaires have been used to compare the usefulness of gastrectomy procedures and to evaluate the postoperative QOL. For this purpose, existing general-purpose disease or symptom specific QOL questionnaires, such as GSRS[26,31,32], Gastrointestinal Quality of Life Index[12,33] and European Organization for Research and Treatment of Cancer Quality of Life Questionnaire; EORTC QLQ-C30 + QLQ-STO22[10,13,18,19,21,34-36], which were established for other purposes and had verified reliability and validity, have been mainly used, because there

have been no established questionnaires specified for the postgastrectomy evaluation. However, these questionnaires are likely to be inadequate for the clinical evaluation of postgastrectomy patients, because they do not contain "dumping" and/or "meal-related distress", which are symptoms that are well-recognized as significantly affecting the postoperative QOL of gastrectomy patients. Actually, our previous study showed that comparison of the influence of the 15 symptom items of the GSRS and 8 symptom items of the original PGSAS, including dumping symptoms and meal-related distress, on the living status and QOL revealed that the effect sizes of the items on the original PGSAS on most main outcome measures were much larger than those of the GSRS items[24]. Therefore, we consider that it is necessary to use questionnaires containing both "dumping" and "meal-related distress" symptoms established for postgastrectomy evaluation (such as PGSAS-45[24]) in future studies of the QOL after gastrectomy.

A limitation of this study was that there might also be unknown clinical factors, in addition to the predictor variables used in the present analysis, which could affect the QOL of postgastrectomy patients. It is necessary to continue to try to find and manage such possible factors by closely observing the living status of gastrectomized patients. The presence, if any, of a strong correlation among the predictor variables used in MRA would cause statistical instability due to multicollinearity, leading to a reduction in the reliability of MRA. Therefore, variance inflation factor (VIF), which is an indicator of multicollinearity, was calculated for the predictor variables used in the study. The VIF values in the MRA shown in Tables 4-6 were 1.0-1.3, 1.0-1.4 and 1.4-2.3, respectively, indicating the absence of any multicollinearity.

Improvement in the gastrectomy procedures to reduce PGS is extremely important and continual efforts to improve the gastrectomy procedures are also necessary in the future. However, it is difficult to eliminate PGS only by improving the gastrectomy procedures. Therefore, attention must be paid to the other clinical factors that have been found to decrease the QOL after gastrectomy and it is necessary to try to improve the lives of postgastrectomy patients in a composite manner by, for example, sufficient surveillance and care for PGS in outpatient practice after gastrectomy. Paying attention to "symptoms" (in particular, "dumping", "meal-related distress", "abdominal pain" and "esophageal reflux", which greatly affect the postoperative QOL), "ability for working" and "necessity for additional meals" to detect these abnormalities early and providing appropriate management and treatment in outpatient practice after surgery would be expected to contribute to the improvement of the postoperative QOL of gastrectomy patients.

ACKNOWLEDGMENTS

The results of this study were presented at Digestive Disease Week 2013, Orland, United States[37].

This study was completed by 52 institutions in Japan. The authors thank all physicians who participated in this study and the patients whose cooperation made this study possible. The contributor of each institution is listed below.

Masanori Terashima (Shizuoka Cancer Center), Junya Fujita (Toyonaka Municipal Hospital), Kazuaki Tanabe (Hiroshima University), Nobuhiro Takiguchi (Chiba Cancer Center), Masazumi Takahashi (Yokohama Municipal Citizen's Hospital), Kazunari Misawa (Aichi Cancer Center Hospital), Koji Nakada, Norio Mitsumori (The Jikei University School of Medicine), Hiroshi Kawahira (Graduate School of Medicine, Chiba University), Tsutomu Namikawa (Kochi Medical School), Takao Inada (Tochigi Cancer Center), Hiroshi Okabe (Kyoto University Graduate School of Medicine), Takashi Urushihara (Hiroshima Prefectural Hospital), Yoshiyuki Kawashima (Saitama Cancer Center), Norimasa Fukushima (Yamagata Prefectural Central Hospital), Yasuhiro Kodera (Nagoya University Graduate School of Medicine), Takeyoshi Yumiba (Osaka Kosei-Nenkin Hospital), Hideo Matsumoto (Kawasaki Medical School), Akinori Takagane (Hakodate Goryoukaku Hospital), Chikara Kunisaki (Yokohama City University Medical Center), Ryoji Fukushima (Teikyo University School of Medicine), Hiroshi Yabusaki (Niigata Cancer Center Hospital), Seshimo Akiyoshi (Tokyo Women's Medical University), Naoki Hiki (Cancer Institute Hospital), Keisuke Koeda (Iwate Medical University), Mikihiro Kano (JA Hiroshima General Hospital), Yoichi Nakamura (Toho University Ohashi Medical Center), Makoto Yamada (Gifu Municipal Hospital), Sang-Woong Lee (Osaka Medical College), Shinnosuke Tanaka (Fukuoka University School of Medicine), Akira Miki (Kobe City Medical Center General Hospital), Masami Ikeda (Asama General Hospital, Yokosuka General Hospital Uwamachi), Satoshi Inagawa (University of Tsukuba), Shugo Ueda (Kitano Hospital), Takayuki Nobuoka (Sapporo Medical University School of Medicine), Manabu Ohta (Hamamatsu University school of Medicine), Yoshiaki Iwasaki (Tokyo Metropolitan Cancer and Infectious diseases Center Komagome Hospital), Nobuyuki Uchida (Haramachi Red-cross Hospital), Eishi Nagai (Graduate School of Medical Sciences, Kyushu University), Yoshikazu Uenosono (Kagoshima University Graduate School of Medicine), Shinichi Kinami (Kanazawa Medical University), Yasuhiro Nagata (National Hospital Organization Nagasaki Medical Center), Masashi Yoshida (International University of Health and Welfare, Mita Hospital), Keishiro Aoyagi (School of Medicine Kurume University), Shuichi Ota (Osaka Saiseikai Noe hospital), Hiroaki Hata (National Hospital Organization, Kyoto Medical Center), Hiroshi Noro (Otemae Hospital), Kentaro Yamaguchi (Tokyo

Women's Medical University Medical Center East), Hiroshi Yajima (The Jikei University Kashiwa Hospital), Toshikatsu Nitta (Shiroyama Hospital), Tsuyoshi Etoh (Oita University), Chikashi Shibata (Tohoku University Graduate School of Medicine).

COMMENTS

Background

Various clinical problems called postgastrectomy syndrome (PGS) occur after gastrectomy, which can interfere with the quality of life (QOL) of gastrectomized patients. To detect potential clinical factors affecting QOL after gastrectomy may improve prevention and management of PGS.

Research frontiers

Several previous studies investigated that the type of gastrectomy procedures affect QOL after gastrectomy. However, other clinical factors affecting postgastrectomy QOL are poorly understood. The research hotspot is to detect potential clinical factors other than type of gastrectomy procedures which affecting postgastrectomy QOL in large population of gastrectomized patients by multivariate analysis using newly developed postgastrectomy syndrome assessment scale (PGSAS)-45.

Innovations and breakthroughs

Several clinical factors such as symptom severity, ability for working and necessity for additional meals had significant impact on the postoperative QOL with considerable effect sizes, while the influence of the extent of gastrectomy was unexpectedly small.

Applications

Paying attention to "symptoms", "ability for working" and "necessity for additional meals" may help to detect PGS early and provide appropriate management and treatment in outpatient practice, which in turn would be expected to improve the QOL in patients after gastrectomy.

Terminology

PGS is an organic, functinal, nutritional or metabolic problems after gastrectomy, which accompanying various symptoms, restriction of food intake, weight loss or decrease in physical activity, and can interfere with the QOL of gastrectomized patients.

Peer-review

This is a well-written paper to analyze the factors affecting the QOL after gastrectomy using PGSAS-45. The authors analyzed postoperative factors using newly developed scale to improve the QOL that underwent gastrectomy for gastric cancer. This paper has potentially important clinical implications.

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P- Reviewer: Endo S, Garcia-Olmo D, Kim HH, Namikawa T
S- Editor: Qi Y **L- Editor**: A **E- Editor**: Zhang FF

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DOI: 10.3748/wjg.v22.i40.8991

World J Gastroenterol 2016 October 28; 22(40): 8991-8998
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

ORIGINAL ARTICLE

Prospective Study

Oropharyngeal acid reflux and motility abnormalities of the proximal esophagus

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Author contributions: Passaretti S designed the study, analyzed manometric data and did the statistical analysis; Mazzoleni G did examinations, analyzed pH-metric data and wrote the paper; Vailati C did examinations and collected data; Testoni PA revised the manuscript; all authors approved the final version.

Institutional review board statement: The study was reviewed and approved by the medical ethics committee of San Raffaele Scientific Institute.

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

Conflict-of-interest statement: All authors have no competing financial, professional or personal interests that might have influenced the performance or presentation of the study.

Data sharing statement: The technical appendix, statistical code and dataset are available from the corresponding author at passaretti.sandro@hsr.it. Participants gave informed consent for data sharing. No additional data are available.

Manuscript source: Invited manuscript

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Received: June 6, 2016
Peer-review started: June 11, 2016
First decision: July 29, 2016
Revised: August 29, 2016
Accepted: September 28, 2016
Article in press: September 28, 2016
Published online: October 28, 2016

Abstract

AIM

To investigate the relationship between pathological oropharyngeal (OP) acid exposure and esophageal motility in patients with extra-esophageal syndromes.

METHODS

In this prospective study we enrolled consecutive outpatients with extra-esophageal symptoms suspected to be related to gastroesophageal reflux disease (GERD). We enrolled only patients with a reflux symptom index (RSI) score-higher than 13 and with previous lung, allergy and ear, nose and throat evaluations excluding other specific diagnoses. All patients underwent 24-h OP pH-metry with the Dx probe and esophageal high-resolution manometry (HRM). Patients were divided into two groups on the basis of a normal or pathological pH-metric finding (Ryan Score) and all manometric characteristics of the two groups were compared.

RESULTS

We examined 135 patients with chronic extra-esophageal syndromes. Fifty-one were considered eligible for the study. Of these, 42 decided to participate in the protocol. Patients were divided into two groups on the basis of normal or pathological OP acid exposure. All the HRM parameters were compared for the two groups. Significant differences were found in the median upper esophageal sphincter resting pressure

(median 71 mmHg *vs* 126 mmHg, P = 0.004) and the median proximal contractile integral (median 215.5 cm•mmHg•s *vs* 313.5 cm•mmHg•s, P = 0.039), both being lower in the group with pathological OP acid exposure, and the number of contractions with small or large breaks, which were more frequent in the same group. This group also had a larger number of peristaltic contractions with breaks in the 20 mmHg isobaric contour (38.7% *vs* 15.38%, P < 0.0001).

CONCLUSION

In patients with suspected GERD-related extra-esophageal syndromes pathological OP acid exposure was associated with weaker proximal esophageal motility.

Key words: Esophagus; Motility; Oropharyngeal reflux; Gastroesophageal reflux disease; High resolution manometry; pH-metry

Core tip: A new oropharyngeal (OP) pH probe now available is more sensitive than traditional pH sensors for faithfully monitoring the pH of OP reflux, and the latest high-resolution esophageal manometry offers a major advance in defining esophageal motility abnormalities compared to conventional manometry. This study compares these two techniques, for the first time, and indicates that in patients with extra-esophageal syndromes pathological OP acid exposure is associated with weaker proximal esophageal motility.

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INTRODUCTION

Gastro-esophageal reflux disease (GERD) is very common in Western countries, with a prevalence of typical manifestations of 10%-20%[1]. In the last few years, ear, nose and throat (ENT) specialists have increasingly attributed a range of atypical manifestations to GERD. In 2006 an international Consensus Group developed a global classification of GERD manifestations, grouping them as either esophageal or extra-esophageal syndromes[1].

It is hard to calculate the prevalence of extra-esophageal syndromes because of their multifactorial etiology and the difficulty of establishing a clear cause-effect relationship between reflux and symptoms[2]. The "gold standard" for determining a pathological gastro-esophageal reflux (GER), 24-h esophageal pH-impedance, is not totally reliable for the diagnosis of laryngopharyngeal reflux (LPR) because the standard impedance probes do not have channels reaching the upper esophageal sphincter (UES) and pharynx and traditional pH sensors are poorly reliable when positioned in the hypopharynx[3].

A new oropharyngeal (OP) pH probe, the Dx probe, is now available, and is more sensitive than traditional pH sensors for faithfully monitoring the pH in the oropharynx[4-6]. It is still not clear why in some patients the GER is limited to the distal esophagus while in others it extends to the proximal esophagus and above the UES, where it can cause extra-esophageal manifestations by a direct mechanism[7]. In order to assess whether esophageal motility plays a role in the proximal extension of reflux, various studies have examined patients with extra-esophageal symptoms using 24-h esophageal pH-metry (some also with a proximal pH-probe) and conventional esophageal manometry, but results have been discordant[8-11]. Esophageal high-resolution manometry (HRM) records esophageal motility more reliably than conventional manometry[12].

The aim of this study in patients with extra-esophageal syndromes was to assess, for the first time, the relationship between pathological OP acid exposure, examined with the Dx probe, and esophageal motor characteristics, assessed by HRM.

MATERIALS AND METHODS

Patients and study design

From October 2011 to March 2013 we prospectively enrolled consecutive patients referred to the gastroenterology outpatient unit of our tertiary center for chronic (> 6 mo) suspected GERD-related extra-esophageal syndromes[1]. In order to increase the probability of identifying a subgroup of patients in this population with pathological OP acid exposure, we enrolled only patients with a reflux symptom index (RSI) score higher than 13[13,14] and with previous lung, allergy and ENT evaluations excluding other specific diagnoses. Other exclusion criteria were: history of thoracic or gastric surgery, dysphagia and known esophageal motility disorders. All the patients underwent 24-h OP pH-monitoring with the Dx probe and esophageal HRM. We compared all the esophageal motility parameters with the OP pH-metry profile.

The protocol was approved by the hospital's medical ethics committee. Informed consent was obtained for procedures and for data management for scientific purposes.

RSI questionnaire

The RSI is a self-administered nine-item questionnaire for symptoms assessment in patients with suspected LPR. The score for each item ranges from 0 (no problem) to 5 (severe problem), up to a maximum of 45[13]. Schindler *et al*[14] developed the validated Italian form of the RSI, which is easily administered, highly

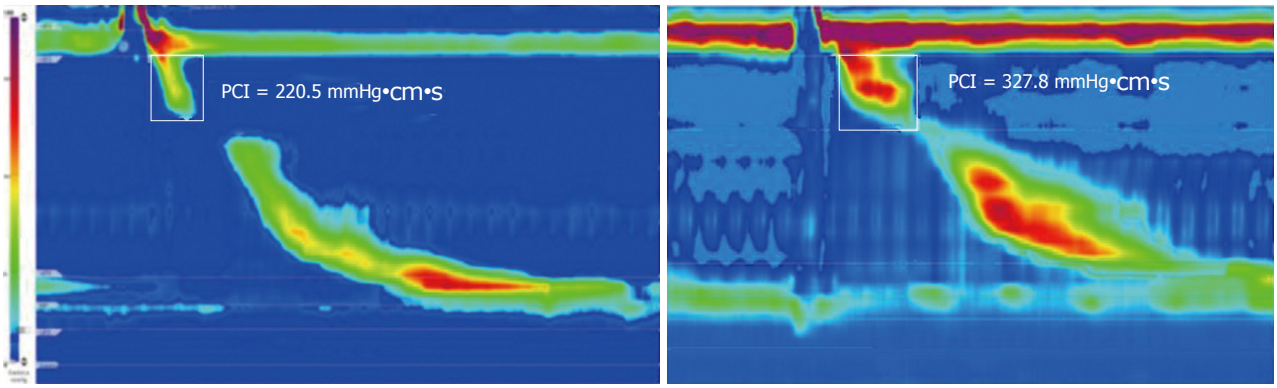

Figure 1 The proximal contractile integral. The proximal contractile integral (PCI) is calculated applying the same algorithm used for the distal contractile integral to quantify contractile pressure exceeding 20 mmHg in the region outlined by the white line. The high- resolution manometry tracing on the left refers to a patient with pathological oropharyngeal (OP) acid exposure (weaker PCI = lighter colors), while the tracing on the right refers to a patient with normal OP acid exposure (stronger PCI = darker color).

reproducible, and ensures excellent clinical validity.

Oropharyngeal pH-monitoring

For OP pH-monitoring we used the Dx-pH measurement system (Restech - Respiratory Technology Corporation, San Diego, CA, United States). The Dx sensor was calibrated in pH 7 and 4 buffer solutions before use. The probe was inserted transnasally and positioned so that the flashing light-emitting diode at its tip was 5-10 mm below the uvula[5]. Patients were asked to keep a diary during the recording period, indicating the times they spent sleeping or orthostatic and the times when they ate or drank and brushed their teeth; these periods were excluded from the analysis. After the 24-h recording the data were downloaded to a dedicated software program (DataView Lite V3, Respiratory Technology Corporation) and pH tracings were all assessed by a single operator (SP) (GM), who was blinded to the manometric results. Ryan Scores were calculated for the supine and upright positions; these composite scores use 5.0 and 5.5 pH thresholds respectively and combine three parameters: (1) the number of reflux episodes; (2) the duration of the longest episode; and (3) the percentage of time below the defined threshold. Scores higher than 9.41 in the upright position and/or higher than 6.81 in the supine position denoted pathological OP acid exposure[6].

HRM

Manometric studies were done using a system (Solar GI HRM, Medical Measurement System, The Netherlands) with a catheter with 36 circumferential solid-state pressure sensors spaced at 1-cm intervals (UniTip High Resolution Kateter 12F, Unisensor, Attikon, Switzerland). Patients fasted overnight then the catheter was placed transnasally, positioned to record from the hypopharynx to the stomach. The manometric protocol was done with patients supine and consisted of a 5-min period to assess basal sphincter pressures, and ten 5-mL water swallows[15-17]. Data were analyzed by a SP, who was blinded to the results of pH tracings, using a dedicated software program (Medical Measurement System Database Software, V8.23a, The Netherlands). All manometric parameters were calculated for each swallow and the contraction patterns were classified according to the Chicago Classification v3.0[16]. The metrics analyzed included: sphincters lengths and resting pressures, lower esophageal sphincter (LES) integrated relaxation pressure (IRP-4s) and distal contractile integral (DCI), as previously defined[15-17]. We also calculated the proximal contractile integral (PCI, Figure 1), applying the same algorithm as for the DCI, to quantify contractile pressure exceeding 20 mmHg for the region spanning from the lower border of the UES to the transition zone (TZ)[18-20]. The individual swallow patterns were classified as peristaltic, premature (distal latency-DL < 4.5 s), hypercontractile (DCI > 8000 mmHg•s•cm), failed (DCI < 100 mmHg•s•cm), weak (DCI < 450 mmHg•s•cm), and fragmented contraction (defect in the 20-mmHg isobaric contour of the peristaltic contraction > 5 cm)[16]. We also evaluated, according to Chicago Classification v2.0, contractions with small defects (between 2 and 5 cm long)[15].

Statistical analysis

For demographic and clinical characteristics we used a parametric analysis with Student's *t* (Table 1) or Fisher's exact test (Figure 2) to test the significance of differences. For metrics regarding esophageal sphincters and the strength of esophageal contraction (Table 2) first we tested the data distribution with the Kolmogorov-Smirnov test. As the data were not normally distributed we used the median, 95% confidence interval and Mann-Whitney *U* test for independent samples. For the contraction patterns (Table 3) we used the chi-square test to analyze the differences between the two groups, considering all the subtypes of pattern. As this test gave a significant result (chi-square 26.8, $P = 0.0001$) we were authorized to make multiple comparisons between

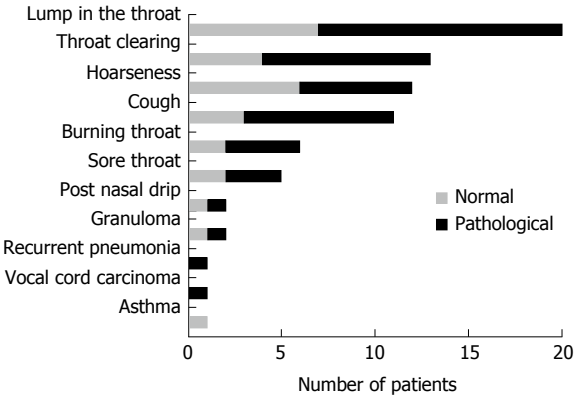

Figure 2 Clinical manifestations of the study population. Number of patients with normal or pathological oropharyngeal acid exposure. Differences between groups are not statistically significant.

Table 1 Patients' main clinical characteristics

	OP pH-	OP pH+
No. of patients	18	24
Male/female (% of male)	8/10 (44%)	6/18 (25%)
Mean age (years ± SD)	52.52 ± 11.71	50.51 ± 14.73
Mean BMI (± SD)	24.50 ± 3.71	24.32 ± 3.61
Mean RSI score	18.00	17.80
No. of patients with typical esophageal symptoms	8/18 (44.44%)	9/24 (37.50%)

Normal (OP pH-) or pathological (OP pH+). OP: Oropharyngeal; RSI: Reflux symptom index; BMI: Body mass index.

Table 2 Esophageal sphincters and strength of contractions

	OP pH-	OP pH+	*P* value
UES length (cm)	4.2 (4.0-4.7)	4.3 (4.1-4.8)	
UES resting pressure (mmHg)	126.0 (96.3-59.7)	71.0 (60.8-110.6)	< 0.05
PCI (cm•mmHg•s)	313.5 (243-489)	215.5 (103-290)	< 0.05
DCI (cm•mmHg•s)	2612 (1121-3195)	1540 (951-2921)	
LES length (cm)	4.65 (3.8-5.1)	5.15 (4.1-5.5)	
LES resting pressure (mmHg)	28.0 (19.8-34.1)	26.0 (20.8-30.8)	
LES 4s-IRP (mmHg)	12.6 (8.0-17.4)	14.2 (11.4-19.1)	

Data are presented as median and 95%CI. UES: Upper esophageal sphincter; PCI: Proximal contractile integral; DCI: Distal contractile integral; LES: Lower esophageal sphincter; IRP: Integrated relaxation pressure.

each subtype of contraction using Fisher's exact test. Probability < 5% was considered significant.

RESULTS

We evaluated 135 patients with chronic extra-esophageal syndromes. Fifty-one were considered eligible. Of these, 42 decided to participate in the protocol; Figure 2 summarizes their main clinical manifestations.

Patients were divided into two groups on the basis of a normal (OP pH-) or pathological (OP pH+) OP acid exposure. The clinical characteristics of the two groups did not significantly differ (Table 1 and Figure 2). All the HRM parameters for the two groups are compared in Tables 2 and 3. Significant differences were found between the two groups in the median UES resting pressure and the median PCI, both lower in patients with pathological OP acid exposure, and the number of contractions with small and large breaks, which were more frequent in the same group.

Table 3 Contraction patterns

	OP pH-	OP pH+	*P* value
No. of correct swallows	184	240	
No. of peristaltic contractions (without breaks)	154/182 (84.61%)	144/235 (61.27%)	< 0.01
No. of failed contractions	2/184 (1.08%)	5/240 (2.08%)	
No. of peristaltic contractions with small breaks	21/182 (11.95%)	68/235 (28.93%)	< 0.01
No. of peristaltic contractions with large breaks	7/182 (3.84%)	23/235 (9.78%)	< 0.01
No. of premature contractions	0	0	
No. of rapid contractions	0	0	

Normal (OP pH-) or pathological (OP pH+). OP: Oropharyngeal.

DISCUSSION

LPR has been diagnosed increasingly frequently in recent years, but often only on the basis of aspecific laryngoscopic findings, common in asymptomatic people too[21,22]. This over-diagnosis poses an important economic burden for the assessment and treatment of these patients, which often unsatisfactory[23]. *Ex adiuvantibus* therapy, with double- dose proton pump inhibitors for long periods (3-6 mo), often achieves a partial response due to the placebo effect or to the multifactorial etiology of these symptoms[7,24]. Regrettably, 24-h pH-impedence is not reliable for the diagnosis of LPR because the standard impedance probes do not have channels reaching the UES and pharynx and traditional pH sensors are poorly reliable when positioned in the hypopharynx. In particular, traditional pH sensors, when positioned in the hypopharynx, are prone to drying out and may cause pseudo-reflux due to artifacts[3].

Recently, two new devices that overcome these limitations have been introduced for the detection of LPR: OP pH-metry (Respiratory Technology Corp.)[4-6] and hypopharyngeal multichannel intraluminal impedence (Sandhill Scientific Inc.)[25,26]. We used the OP Dx probe to detect acid reflux in the oropharynx

of patients with clinically suspected LPR. This sensor measures the pH of both liquid and aerosolized droplets in the posterior oropharynx, avoids drying, does not require contact with fluid or tissue for electrical continuity and has a teardrop shape with the sensor oriented downward to avoid becoming covered with food or mucus[5]. The Dx probe is more sensitive than traditional pH monitoring for detecting LPR[4]. It can not distinguish healthy volunteers from subjects with laryngeal and reflux symptoms[27], but it can identify patients who respond to medical or surgical treatment of GERD[28,29].

We considered OP acid exposure as normal or pathological according to Ryan scores. These composite scores were calculated by Ayazi *et al*[6] using the pH thresholds that are best for defining abnormal OP pH.

In this study we included patients with clinically suspected LPR, *i.e.* with extra-esophageal symptoms and a RSI score higher than 13 and with previous lung, allergy and ENT evaluations excluding other causes of symptoms. In this population we could identify a subgroup in which a pathological LPR was objectively established by 24-h OP pH-monitoring. As far as we know, it is still not clear why some patients have GER limited to the distal esophagus while in others it extends to the proximal esophagus and above the UES. Seeking an answer to this question, different studies have used conventional manometry to assess esophageal motor function in GERD patients. In patients with typical syndromes esophago-gastric junction (EGJ) impairment and ineffective esophageal motility (IEM) were strongly implicated in the development of GERD[30] and the prevalence of these abnormalities rose with the severity of the reflux disease[31]. In contrast, there are few and discordant data about motility abnormalities in patients with extra-esophageal syndromes[8-10,32]. Fouad *et al*[8] found IEM significantly more often in patients with GERD and chronic cough (41%) or asthma (53%) and numerically more often in patients with GERD and laryngitis (31%) than in patients with heartburn (19%). DiBaise *et al*[9] reported no significant difference in motility parameters between GERD patients with typical symptoms and those with extra-esophageal symptoms alone. Patti *et al*[10] reported that in patients who had pH < 4 in the proximal esophagus for more than 3% of the time, the LES was weaker and shorter and UES pressures and peristalsis amplitude lower.

HRM offers a major advance in defining esophageal motility abnormalities[12]. It employs numerous closely spaced pressure sensors, which overcomes the problem of movement-related artifacts for esophageal sphincters and can reveal the segmental character of esophageal peristalsis and the anatomy of the EGJ. Daum *et al*[33] showed that the frequency of esophageal dysmotility in GERD patients was higher using HRM than conventional manometry.

There are only few HRM studies so far in patients with extra-esophageal syndromes[34,35] and most have been done on patients who had an indirect diagnosis of LPR, based on clinical manifestations, positive response to antisecretory therapy or pathological esophageal pH (more frequently) or pH-impedance monitoring, which is not altogether reliable for detecting LPR. As we discussed above, in most cases the motility has not really been assessed in a population with established LPR. We studied patients with extra-esophageal syndromes using OP pH-monitoring and HRM in order to find out whether there was a motility pattern characteristic of patients with established pathological LPR. Objective identification of LPR is essential to define a population of true patients in which the LPR is proven, not just assumed. We compared all the motility parameters that can be obtained with HRM for patients with pathological OP acid exposure and those with a normal result. The aim of this study was to correlate HRM and OP pH-metry and this is the first comparison of the two techniques. The study did not aim to assess the correlation between manometric features and extra-esophageal syndromes.

The parameters that differed significantly in the two groups were the median UES resting pressure, the median PCI and the number of contractions with small or large breaks. All these parameters are hard to assess with conventional manometry[12,33]. The median UES resting pressure was significantly lower in patients with pathological OP acid exposure. UES incompetence is necessary for LPR[36,37]. A recent study showed that LPR (video-endoscopically documented) could be induced by slow esophageal liquid infusion in patients with a clinical diagnosis of GERD-related extra-esophageal syndromes but not in healthy controls, and that the application of 20-30 mmHg cricoid pressure significantly raised UES intraluminal pressure and prevented the LPR[38]. A new, individually fitted UES assist device (Reza-Band®) to be worn at night has been recently marketed and seems to prevent LPR.

Even though the PCI metric is not included in the Chicago Classification, it has been evaluated in a few published studies, including patients with extra-esophageal symptoms[18-20]. We found it was significantly lower in the group with pathological OP acid exposure. Possibly, therefore, lower proximal esophageal contractile function may lead to less reflux clearance, which would allow the reflux to extend proximally. Clearly, however, it is also possible that a reflux with proximal extension may lead to impairment of upper esophageal motility.

Finally, patients with pathological OP acid exposure had significantly more contractions with small or large breaks in the 20 mmHg isobaric contour between UES and EGJ. The Chicago Classification 2012 distinguishes small (2-5 cm long) and large (> 5 cm) breaks as subtypes of weak peristalsis[15], while the HRM Working Group for Chicago Classification v3.0 proposes considering small breaks as normal and only large breaks as fragmented contractions[16]. However, in our series both types of break were significantly

more frequent in the group with pathological OP acid exposure. The significantly larger number of contractions with breaks in these patients might conceivably result in ineffective reflux clearance[39]. The low- pressure segments anatomically correspond to the TZ from striated to smooth esophageal muscle, where the muscle types are imbricated[40]; an area of extreme hypotensive peristalsis correlates with incomplete bolus transit[41]. HRM combined with multichannel impedance may help clarify when the manometric characteristics in patients with pathological OP acid exposure are really associated with delayed reflux clearance. A recent study using this technique in patients with typical GERD reported that those with a pathological number of large breaks had significantly slower reflux clearance (BCT) in the supine position and longer acid exposure time[42].

The motility features of the distal esophagus (DCI, LES resting pressure and 4s-IRP) did not significantly differ in the two groups in this study but this is not really surprising; even in patients with normal distal reflux, the lack of effective proximal reflux clearance might allow a small amount of reflux to flow up from the distal esophagus to the larynx and pharynx, where even a single episode of LPR is considered pathological[25,43].

In conclusion, this study compared, for the first time, the results of OP pH monitoring and esophageal HRM. We found a significant correlation between pathophysiological features, particularly pathological OP acid exposure and esophageal motility. Further studies are now needed to establish whether the motility characteristics we found in patients with pathological OP acid exposure are the cause or consequence of pathological acid reflux.

COMMENTS

Background

In the last few years many extra-esophageal manifestations have been increasingly attributed to gastro-esophageal reflux (GER) and laryngopharyngeal reflux (LPR). It is not clear why in some patients GER is limited to the distal esophagus while in others it extends above the upper esophageal sphincter (UES).

Research frontiers

A current hotspot is to define the cause-effect relationship between LPR and extra-esophageal syndromes since their diagnosis and treatment pose an important economic burden.

Innovations and breakthrough

A new oropharyngeal (OP) pH probe is now available which is more sensitive than traditional pH sensors for faithfully monitoring the pH of OP reflux, and high-resolution esophageal manometry offers a major advance in defining esophageal motility abnormalities compared to conventional manometry. This is the first study comparing the two techniques.

Applications

The evidence of a correlation between pathological OP acid exposure and weak (altered) esophageal motility could change future therapeutic strategies.

Terminology

According to the Montreal classification of gastro-esophageal reflux disease (GERD), the manifestations of the disease are divided into esophageal and extra-esophageal syndromes. Extra-esophageal syndromes are further divided into syndromes with an established association with GERD (cough, laryngitis, asthma, dental erosion) and syndromes with a proposed association with GERD (pharyngitis, sinusitis, idiopathic pulmonary fibrosis, recurrent otitis media). "LPR" is the term used to define the reflux of gastric content through the esophagus, reaching the upper UES and pharynx.

Peer-review

This study is deemed worthwhile since the authors investigated the relationship between pathological acid exposure and esophageal motility in patients with extra-esophageal syndromes suspected to be related to GERD. The authors also suggest that pathological OP acid exposure is associated with weaker proximal esophageal motility in patients with suspected GERD-related extra-esophageal syndromes. Although the results and discussions will satiate the readers' interest, I have some comments mentioned below.

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P- Reviewer: Goda K, Lakatos PL **S- Editor**: Gong ZM
L- Editor: A **E- Editor**: Zhang FF

World Journal of Gastroenterology

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DOI: 10.3748/wjg.v22.i40.8999

World J Gastroenterol 2016 October 28; 22(40): 8999-9011
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

Systematic review: Safety of balloon assisted enteroscopy in Crohn's disease

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Supported by Training Grant Through the National Institute of Diabetes and Digestive and Kidney Diseases, No. 5T32DK007202 to Dulai PS.

Conflict-of-interest statement: All authors have no relevant conflicts of interest.

Data sharing statement: There are no additional data.

Manuscript source: Unsolicited manuscript

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Received: May 18, 2016
Peer-review started: May 18, 2016
First decision: June 20, 2016
Revised: July 1, 2016
Accepted: August 1, 2016
Article in press: August 1, 2016
Published online: October 28, 2016

Abstract

AIM

To determine the overall and comparative risk of procedure related perforation of balloon assisted enteroscopy (BAE) in Crohn's disease (CD).

METHODS

Systematic review (PROSPERO #CRD42015016381) of studies reporting on CD patients undergoing BAE. Seventy-three studies reporting on 1812 patients undergoing 2340 BAEs were included. Primary outcome of interest was the overall and comparative risk of procedure related perforation of diagnostic BAE in CD. Secondary outcomes of interest were risk of procedure related perforation of diagnostic double balloon enteroscopy (DBE), risk of procedure related perforation of therapeutic BAE, efficacy of stricture dilation, and clinical utility of endoscopically assessing small bowel disease activity.

RESULTS

Per procedure perforation rate of diagnostic BAE in CD was 0.15% (95%CI: 0.05-0.45), which was similar to diagnostic BAE for all indications (0.11%; IRR = 1.41, 95%CI: 0.28-4.50). Per procedure perforation rate of diagnostic DBE in CD was 0.12% (95%CI: 0.03-0.44), which was similar to diagnostic DBE for all indications (0.22%; IRR = 0.54, 95%CI: 0.06-0.24). Per procedure perforation rate of therapeutic BAE in CD was 1.74% (95%CI: 0.85-3.55). Eighty-six percent

of therapeutic perforations were secondary to stricture dilation. Dilation was attempted in 207 patients and 30% required surgery during median follow-up of 18 months. When diagnostic BAE assessed small bowel disease activity, changes in medical therapy resulted in endoscopic improvement in 77% of patients.

CONCLUSION
Diagnostic BAE in CD has a similar rate of perforation as diagnostic BAE for all indications and can be safely performed in assessment of mucosal healing.

Key words: Crohn's disease; Balloon; Enteroscopy; Safety; Perforation; Stricture

Core tip: Crohn's disease (CD) affects the small bowel in up to 60% of patients, but evaluation of small bowel disease is often difficult. Balloon assisted enteroscopy (BAE) can evaluate the small bowel but its safety and diagnostic utility is not established. This systematic review includes 73 studies reporting on 1812 patients undergoing 2340 procedures to evaluate its safety and possible utility. We found that diagnostic BAE in CD had a similar rate of perforation as diagnostic BAE for all indications, suggesting BAE is a safe method for small bowel evaluation in CD.

Arulanandan A, Dulai PS, Singh S, Sandborn WJ, Kalmaz D. Systematic review: Safety of balloon assisted enteroscopy in Crohn's disease. *World J Gastroenterol* 2016; 22(40): 8999-9011 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i40/8999.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i40.8999

INTRODUCTION

Crohn's disease (CD) is a debilitating chronic inflammatory bowel disease (IBD), which if left untreated, leads to penetrating complications (strictures, fistulae, abscesses)[1-3]. Frequent disease activity assessment with the intent of adjusting therapy has been demonstrated to significantly reduce the risk of disease related complications[4-10]. Small bowel involvement in CD is reported to occur in up to 60% of patients, with nearly 30% of patients having isolated small bowel disease[11-13]. Conventional upper and lower endoscopy, however, are limited in their ability to assess small bowel disease activity[14-18], and clinical or biochemical markers of disease activity infrequently correlate with small bowel mucosal inflammation[19]. The presence of inflammation cannot be reliably excluded by radiological imaging alone[20], and video capsule endoscopy (VCE) carries a risk for capsule retention in structuring or penetrating disease and it lacks therapeutic capability[21-24].

Balloon assisted enteroscopy (BAE) offers the advantage of providing both diagnostic (mucosal biopsies) and therapeutic (stricture dilation) potential, but its use in CD is currently limited to symptomatic patients with negative ileocolonoscopy, VCE, and/or cross sectional imaging, and the feasibility and diagnostic utility of routine BAE in clinical practice for CD is yet to be established[20,25-29]. Therefore, we performed a systematic review of the literature to quantify the safety and therapeutic utility of this endoscopic technique. We anticipate these data will help to better characterize the growing importance of BAE in CD.

MATERIALS AND METHODS

Data sources and search strategy

The following databases were searched in October 2015: MEDLINE (PubMed, January 1946 to October 3, 2015); Cochrane Central Register of Controlled Trials (Wiley, 2015); and Embase (Embase.com, January 1974 to October 3, 2015). The search included indexed terms and text words to capture the following concepts: CD and balloon enteroscopy. There were no language or study design restrictions. The search strategy was adjusted for the syntax appropriate for each database. The reference lists of included articles and review articles were examined for additional relevant studies. The full search strategy, a priori, is available at the international prospective register of systematic reviews (PROSPERO #CRD42015016381).

Study selection and extraction

Studies were included for analysis if they met the following inclusion criteria: Randomized controlled trials, cohort studies, published meeting abstracts, or case series of 5 or more consecutive patients with CD, undergoing BAE for diagnostic or therapeutic purposes. Review articles and studies with fewer than 5 patients with CD were excluded. Studies with insufficient data for adverse outcomes and follow-up were excluded only after attempting to contact the primary author(s). The included population was patients of all ages with CD undergoing BAE for diagnostic and/or therapeutic purposes with clearly reported adverse outcomes. Comparative studies to assess diagnostic performance, therapeutic utility, and safety of BAE were included.

Two reviewers (Arulanandan A and Dulai PS) independently evaluated each of the articles for eligibility. Inclusion decisions for each article were made independently based on the eligibility criteria, with disagreements being resolved by a third reviewer (Kalmaz D) and consensus. A reviewer (Arulanandan A contacted the primary author(s) as required to obtain any necessary missing data from the original publications and because no language restrictions were applied, publications were translated into English as required. The reviewers followed the Preferred

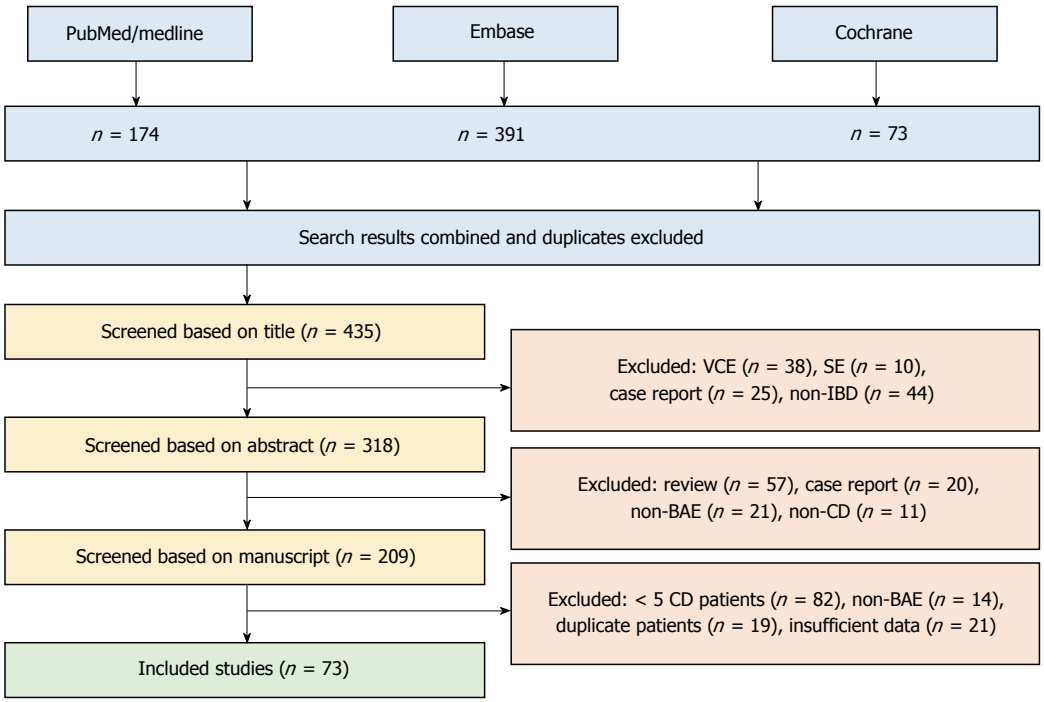

Figure 1 Studies identified and reasons for exclusion. VCE: Video capsule endoscopy; SE: Spiral enteroscopy; IBD: Inflammatory bowel disease; BAE: Balloon-assisted enteroscopy; CD: Crohn's disease.

Reporting Items for Systematic Reviews and Meta-Analyses standards for systematic review.

Outcomes

Our primary outcome of interest was the safety of diagnostic BAE [single (SBE) or double (DBE) balloon enteroscopy) in CD, for which we calculated per procedure perforation rate and compared that to per procedure perforation rate when using diagnostic BAE for all indications. The expected rate of perforation when utilizing diagnostic BAE for all indications was derived from a Japanese database of 29068 patients[30].

Secondary outcome of interest was the safety of diagnostic DBE in CD, for which we calculated per procedure perforation rate and compared that to per procedure perforation rate when using diagnostic DBE for all indications. The expected rate of perforation when utilizing diagnostic DBE for all indications was derived from a systematic review of 9047 DBEs[31]. Other outcomes of interest were risk of procedure related perforation of therapeutic BAE, efficacy of stricture dilation, and clinical utility of endoscopically assessing small bowel disease activity.

Statistical analysis

We used the random-effects model described by DerSimonian and Laird to calculate pooled rates (and 95%CI) of perforation with BAE, stratified by indication (diagnostic *vs* therapeutic)[32]. Due to lack of consistent reporting in multiple studies, we did not perform a quantitative meta-analysis of factors associated with perforation, but rather discussed them qualitatively. The STATA "incidence ratio (IR)" command (version 10.0; STATA, College Station, TX, United States) was used to make comparisons for perforation rates, and the relative rates for perforation were calculated as incidence rate ratios (IRRs).

RESULTS

Patient and study characteristics

Of the 638 studies identified, 73 studies reporting on 1812 CD patients undergoing 2340 BAE procedures (DBE: *n* = 2027, SBE: *n* = 187, BAE not-specified: *n* = 126) were included in the final analysis[28,33-104] (Figure 1, Tables 1 and 2). The majority of studies involved international institutions (85%)[28,33,34,36-40,42-47,51-56,58,61-78,80-93,96,97,99-105], reporting retrospectively (74%)[34,35,37-43,47-51,53-57,59,60,62,63,65,67-69,71-73,75-85,87-91,93-96,99,101,102,104], on their experience with BAE. Of the 1812 patients included in the analysis, 597 (33%) were newly diagnosed with CD and 1215 (67%) had a known diagnosis of CD prior to undergoing BAE. Among the patients with known CD where the indication for the procedure was clearly documented, BAE was performed primarily for monitoring of disease activity (61%), known strictures (16%), or small bowel obstruction (4%). One thousand nine hundred and thirty-eight BAE (83%) were diagnostic and 402 BAE (17%) were therapeutic procedures.

The 3 prospective randomized controlled trials identified in our search reported on a total of 37 CD patients[44,52,61]. Two of these studies compared DBE to SBE and found DBE to have comparable[52] or

Table 1 Study demographics *n* (%)

	BAE studies, *n* = 73 Patients, *n* = 1812	DBE, *n* = 60 *n* = 1509	SBE, *n* = 11 *n* = 187
Published manuscripts	47 (64)	39 (65)	8 (72)
United States center	11 (15)	9 (15)	0 (0)
Prospective	19 (26)	14 (23)	6 (55)
Published pre-2010	18 (25)	17 (28)	1 (10)
Crohn's patients per study			
5-10	26 (36)	23 (38)	3 (27)
11-25	25 (34)	20 (33)	5 (45)
> 25	22 (30)	17 (28)	3 (27)
Age, mean ± SD	42.9 ± 15.4	42.4 ± 15.5	45.0 ± 15.8

Three studies and 116 patients were solely reported as "balloon assisted enteroscopy"; 1 study included both DBE and SBE. DBE: Double balloon enteroscopy; SBE: Single balloon enteroscopy; BAE: Balloon assisted enteroscopy.

superior efficacy and diagnostic yield[44], while the other suggested that fluoroscopy increases insertion depth[61]. The largest prospective cohort study included 193 patients from 62 endoscopic centers in Germany over a 2-year span and had no perforations in CD patients[64].

Safety of BAE

The rate of perforation with diagnostic BAE in CD (1.5 per 1000 procedures) was similar to that reported when utilizing diagnostic BAE for all indications (1.1 per 1000 procedures, IRR = 1.41, 95%CI: 0.28-4.50). The rate of perforation with diagnostic DBE in CD (1.2 per 1000 procedures) was similar to that reported when utilizing diagnostic DBE for all indications (2.2 per 1000 procedures, IRR = 0.54, 95%CI: 0.06-2.24). The rate of perforation with therapeutic BAE in CD was 1.74% (Tables 3 and 4).

Among 1812 patients who underwent a total of 2340 procedures, there were 8 reported perforations with DBE (per procedure 0.39%, 95%CI: 0.12-0.66, per patient 0.53%, 95%CI: 0.16-0.90)[33-35,48,49,85,91,94,96], 1 reported perforation with SBE (per procedure and per patient 0.53%, 95%CI: 0.0-1.57)[96], and 1 perforation with BAE not otherwise specified. Of the 8 DBE perforations, 5 were during dilation of de novo CD strictures and the other 3 were during diagnostic evaluations of an adhesion site, an anastomosis stricture, and a site that was not specified. The 1 SBE perforation was during dilation of a de novo CD stricture site. This equated to an overall per procedure and per patient perforation rate of 0.43% (95%CI: 0.16-0.67) and 0.55% (95%CI: 0.21-0.89), respectively, for BAE in CD including both diagnostic and therapeutic procedures[94]. There was minimal heterogeneity among studies (I^2 = 0).

Follow-up and impact of BAE on medical and surgical management

Follow-up after BAE was reported in 407 patients with a median follow-up period of 18 mo (range 10-70)[28,33-40,85,86,88,91] (Figure 2). Stricture dilation was performed in 171 patients, resulting in 5 perforations (all at de novo CD stricture sites) and 166 technically successful dilations. Dilation was not attempted in 36 patients due to: inability to insert scope up to stricture site (*n* = 8), inability to maintain guide wire or through-the-scope balloon at correct position of the stricture (*n* = 5), stricture length or severe angulation (*n* = 4), severe inflammation (*n* = 6), the presence of intra-abdominal adhesions that prohibited advancement of the DBE (*n* = 2), a perforation during overtube advancement at an anastomotic site (*n* = 1), and/or non-obstructing or severely ulcerated strictures (*n* = 10). During follow-up, of the 166 patients where stricture dilation was technically successful, 72 (43%) required repeat stricture dilation and 42 (25%) patients required surgery for persistent symptoms.

Of the remaining 200 patients, 173 had clearly reported outcomes regarding changes in medical therapy and treatment response[28,36,86,88,91]. Based on BAE findings, 139 (80%) patients either initiated therapy with an immunomodulator (azathioprine or methotrexate, *n* = 83), initiated therapy with an anti-tumor necrosis factor agent (*n* = 52), or they were switched from one anti-tumor necrosis factor agent to another (*n* = 4). Seven (4%) patients required surgery, and the remaining either refused a step-up in medical therapy (*n* = 9, 5%) or they had no change in medical therapy needed (*n* = 18, 10%). Of the 139 patients where a change in medical therapy was performed, 92 (66%) achieved clinical remission at the first follow-up (as defined by the CD activity index)[28,36,86,88]. Repeat BAE was performed in 133 patients with a majority achieving endoscopic improvement in disease activity or complete healing of mucosal lesions (62%)[28,36,86,88]. A third follow-up BAE was performed in 113 patients, and rates of complete or nearly-complete mucosal healing improved from 29% (*n* = 33) to 43% (*n* = 57) with changes in therapy based on BAE results[86].

Table 2 Studies involved international institutions on their experience with balloon assisted enteroscopy

Study	Published manuscript	2010-present	United States	Prospective	Mean age	Patients	Procedures	Therapeutic	Perforations
Akarsu *et al*[90]	X	X			47.8	39	39	0	0
Aktas *et al*[95]		X	X		53	58	58	0	0
Aktas *et al*[92]	X	X		X	51	31	31	2	0
Arihiro *et al*[96]		X			56.3	32	32	9	1
Bartel *et al*[41]		X	X		52.6	38	38	0	0
Bartel *et al*[49]		X	X		54.3	7	7	7	1
Bartel *et al*[50]		X	X		62.7	15	15	0	0
Chen *et al*[84]	X	X			51	8	8	0	0
Choi *et al*[82]	X	X			43.5	7	7	0	0
de Ridder *et al*[97]	X	X		X	15	14	14	0	0
Despott *et al*[33]	X			X	46.4	11	13	13	1
Di Caro *et al*[51]	X				52	7	7	0	0
Di Nardo *et al*[98]	X	X		X	13	26	26	5	0
Ding *et al*[85]		X			39	12	22	22	1
Domagk *et al*[52]	X	X		X	52	11	11	0	0
Dutta *et al*[99]	X	X			42	14	14	0	0
Fan *et al*[28]	X	X		X	Not reported	77	308	0	0
Gill *et al*[34]	X	X			52.7	20	20	10	2
Halloran *et al*[35]		X	X		44.8	21	40	40	1
Hirai *et al*[91]	X	X			36	65	110	110	1
Huang *et al*[53]	X				10	7	7	0	0
Jang *et al*[42]	X	X			32.7	24	32	0	0
Jeon *et al*[54]		X			36.4	30	39	0	0
Lakatos *et al*[55]	X				51.6	6	6	0	0
Li *et al*[43]	X				49	13	13	0	0
Liu *et al*[56]	X				8.5	5	5	0	0
Lurix *et al*[57]		X	X		59	5	5	0	0
Maaser *et al*[58]	X	X		X	54.9	59	59	0	0
Mann *et al*[59]		X	X		59	9	9	0	0
Mann *et al*[60]		X	X		59	23	23	0	0
Manner *et al*[61]				X	56	20	20	0	0
Manno *et al*[100]	X	X		X	61	11	11	1	0
May *et al*[44]	X	X		X	53	9	9	0	0
Mensink *et al*[36]	X	X		X	53	50	50	0	0
Milewski *et al*[62]	X			X	45	75	75	12	0
Moreels *et al*[63]		X			Not reported	6	6	0	0
Morise *et al*[89]					13	76	76	0	0
Morishima *et al*[37]	X	X			36	17	35	35	0
Moschler *et al*[64]					35.4	193	193	0	0
Nakano *et al*[47]	X	X		X	64	36	36	36	0
Navaneethan *et al*[94]	X	X			56.8	49	59	9	1
Ohmiya *et al*[38]	X	X	X		41	23	23	23	0
Parker *et al*[65]	X				48	11	11	0	0
Pata *et al*[66]		X			Not reported	16	16	4	0
Peng *et al*[67]	X	X		X	53	15	15	0	0
Pohl *et al*[39]	X				Language[1]	19	21	21	0
Qing *et al*[68]	X				36	7	7	0	0
Rahman *et al*[48]		X			Not reported	55	55	3	1
Roushan *et al*[83]	X	X	X		Not reported	7	7	0	0
Russo *et al*[101]	X	X			47.2	6	6	0	0
Safatle *et al*[93]		X			57	9	9	0	0
Schulz *et al*[69]		X			48.6	11	11	0	0
Seiderer *et al*[70]	X	X			50.8	10	10	0	0
Shen *et al*[71]	X			X	33.9	8	8	0	0
Shi *et al*[72]	X	X			13	35	35	0	0
Sidhu *et al*[73]	X	X			61.2	39	39	0	0
Sun *et al*[74]		X			52	7	7	0	0
Takenaka *et al*[45]	X			X	52	10	10	0	0
Tsujikawa *et al*[102]	X	X		X	31	17	17	7	0
Uchida *et al*[75]	X				48.9	6	9	1	0
Urs *et al*[76]	X	X			12.9	5	5	0	0

Urs *et al*[77]		X			12.7	7	13	0	0
Watanabe *et al*[88]	X	X			10.5	10	20	0	0
Watanabe *et al*[78]		X			Not reported	59	60	0	0
Westerhoff[79]		X			Not reported	18	18	0	0
Wiarda *et al*[46]			X		Not reported	18	18	0	0
Xu *et al*[87]	X	X		X	36	21	21	0	0
Yamada *et al*[40]	X	X			Not reported	46	128	27	0
Yoshida *et al*[103]		X			37	10	10	5	0
Yu *et al*[86]		X		X	Not reported	36	108	0	0
Zhang *et al*[80]	X	X		X	31.6	5	5	0	0
Zhi *et al*[81]	X	X			Language[1]	7	7	0	0
Zhu *et al*[104]	X				36.3	23	23	0	0

[1]Language: Unable to translate full manuscript, data was extracted from abstract which did not disclose age.

Table 3 Diagnostic and therapeutic procedures *n* (%)

	Procedures, *n* = 2340	Perforations, *n* = 10	Rate (%)
Diagnostic BAE	1938 (83)	3 (30)	0.15
Therapeutic BAE	402 (17)	7 (70)	1.70
Diagnostic DBE	1666 (71)	2 (20)	0.12

BAE: Balloon assisted enteroscopy; DBE: Double balloon enteroscopy.

DISCUSSION

In our systematic review of 1812 CD patients undergoing 2340 BAE, the rate of perforation with diagnostic BAE in CD was similar to that seen when utilizing diagnostic BAE for other indications. The rate of perforation with diagnostic DBE in CD was also similar to that seen when utilizing diagnostic DBE for other indications. Additionally, findings from diagnostic BAE in conjunction with changes in medical therapy resulted in improved clinical and endoscopic disease activity in a majority of patients. Diagnostic BAE demonstrated a meaningful change in clinical care with a similar safety profile in CD compared to other indications.

Therapeutic BAE also exhibited significant clinical utility as approximately 70% of patients in our study avoided surgery after stricture dilation, albeit with higher rates of perforation. This increased risk of perforation with stricture dilation in CD[106] must be weighed against the therapeutic benefits achieved. Efforts need to focus on risk stratification of small bowel strictures to determine which patients may be more suitable for small bowel resection as compared to endoscopic dilation. Prior studies have demonstrated that both length and location are useful prognostic factors, and short strictures located in the large bowel or at the site of prior anastomosis are likely to be most amendable to endoscopic dilation as compared to complex or lengthy de-novo strictures[107-109]. Within our study at least three of the six perforations during stricture dilation were at ulcerated sites, and at least two of the perforations were in patients with previous stricture dilations. As the alternative to symptomatic strictures is surgical resection or strictureplasty, our results suggest that stricture dilation *via* BAE can be done in accessible, short-length symptomatic strictures, and it has better performance in anastomotic strictures, but it may need to be avoided in presence of significant CD-related inflammation.

The relative excess risk of endoscopy associated perforations among IBD patients as compared to non-IBD patients has previously been demonstrated, with disease severity and steroid use (a surrogate for disease activity) being two of the strongest predictors for a procedure related perforation[30,110-112]. Within our systematic review, the total rate of perforation with BAE in CD (4.27 per 1000 procedures) when including both diagnostic and therapeutic procedures was nearly 4 times that reported with diagnostic BAE for all indications (1.1 per 1000 procedures), and the significant majority of this risk was seen in therapeutic procedures.

Our results demonstrate that diagnostic BAE is a safe tool in monitoring small bowel disease activity and may have a role in guiding medical treatment to achieve clinical remission and mucosal healing. The rate of perforation with diagnostic BAE in CD (1.55 per 1000) was also similar to that reported with lower endoscopy in IBD patients (1.89 per 1000)[112]. Other modalities have been demonstrated to have a reasonable diagnostic accuracy for assessing small bowel disease activity in CD[22,113-124], but they have several technical and practical limitations that prevent their routine use in clinical practice[48,113,114,121,125-127]. In our pooled analysis, findings from BAE in conjunction with changes in medical therapy resulted in improved clinical and endoscopic disease activity in a substantial majority of patients. Although further prospective studies are needed to understand the positioning of BAE in disease activity assessment and treating to a

Table 4 Balloon assisted enteroscopy cases with procedure related perforation

Study	Demographics	Site and characteristics of perforation	Therapy	Procedure	Outcome after perforation
Despott *et al*[33]	Long standing CD (> 30 yr) with 5 prior SB resections currently on azathioprine and steroids	3 jejunal strictures (2 inflammatory, 1 fibrotic) with severe ulcerations at stricture sites	Dilated to maximum of 16.5 mm	DBE - Technically difficult due to adhesion-related angulations and fixation, and strictures were significantly ulcerated	Perforation diagnosed within 8 h, patient had laparotomy and temporary jejunostomy. Patient made full recovery and jejunostomy was reversed.
Gill *et al*[34]	Retained video capsule in patient with known CD	Non-obstructing jejunal stricture with mild inflammation and ulceration at the stricture site	Dilated to 15 mm	DBE - otherwise not specified	Underwent surgery, outcome otherwise not specified.
Gill *et al*[34]	Known CD patient had previously responded well to dilation up to 15 mm	Distal obstructing ileal stricture with mild inflammation and ulceration at the stricture site	Dilated to 15 mm	DBE - otherwise not specified	Underwent surgery, outcome otherwise not specified.
Halloran *et al*[35]	Known CD patient who had undergone prior surgical resection.	Scarred bowel loop adhesion site	Not specified	DBE - Perforation occurred with overtube advancement and straightening of a scarred bowel loop	Outcome not specified.
Ding *et al*[85]	Known CD patient	SB stricture, otherwise not specified	Dilation related perforation, otherwise not specified	DBE - Dilation related perforation, otherwise not specified	Perforation diagnosed within 12 h, patient had laparotomy and resection with ileostomy.
Bartel *et al*[49]	Retained video capsule in patient with known CD	SB stricture, otherwise not specified	Not specified	DBE - Otherwise not specified	Emergent surgical intervention, otherwise outcome not specified.
Rahman *et al*[48]	Known CD patient	Ulcer at anastomosis site	Not specified	DBE - Perforation directly related to ulcer at anastomosis	Patient made full recovery after surgical resection and primary reanastomosis.
Navaneethan *et al*[94]	Known CD patient	Not specified	Not specified	Not specified	Underwent surgery, outcome otherwise not specified
Arihiro *et al*[96]	Known CD patient	SB stricture, otherwise not specified	Dilation related perforation, otherwise not specified	SBE - Dilation related perforation, otherwise not specified	Patient improved over time without any surgical intervention.
Hirai *et al*[91]	Known CD patient	SB stricture, otherwise not specified	Dilation related perforation, otherwise not specified	DBE - Dilation related perforation, otherwise not specified	Patient had emergency partial ileal resection and made a full recovery.

CD: Crohn's disease; SB: Small bowel; DBE: Double balloon enteroscopy; SBE: Single balloon enteroscopy.

target of mucosal healing in CD, these data suggest that BAE may be a useful tool for assessing small bowel disease activity and treatment response in CD.

Our study has highlighted several key findings but it also has several limitations. There is an inherent selection bias in patients who undergo BAE that undoubtedly affects our results. The majority of analyzed studies were retrospective with variable objectives, different inclusion criteria, and limited follow-up data. Additionally, the lack of prospective studies comparing BAE to cross-sectional imaging in evaluation of small bowel disease makes evaluating the utility of diagnostic BAE difficult. Lastly, BAE has a high rate of incomplete enteroscopy[64], which likely was not portrayed in our results, as completion rates were not consistently documented. Strengths of our study included the extensive search performed and the large number of patients and procedures analyzed.

Diagnostic BAE in CD has a similar perforation rate as diagnostic BAE in other indications and may help safely guide medical therapy via assessment of mucosal healing. Therapeutic BAE may help avoid surgery in patients with symptomatic strictures, but the rate of perforation with therapeutic procedures is higher and thus providers must exercise caution when utilizing this endoscopic technique. Further efforts should focus on risk stratification of patients to ensure optimal safety and diagnostic yield and further studies are needed to identify patient and stricture characteristics at highest risk for procedure related

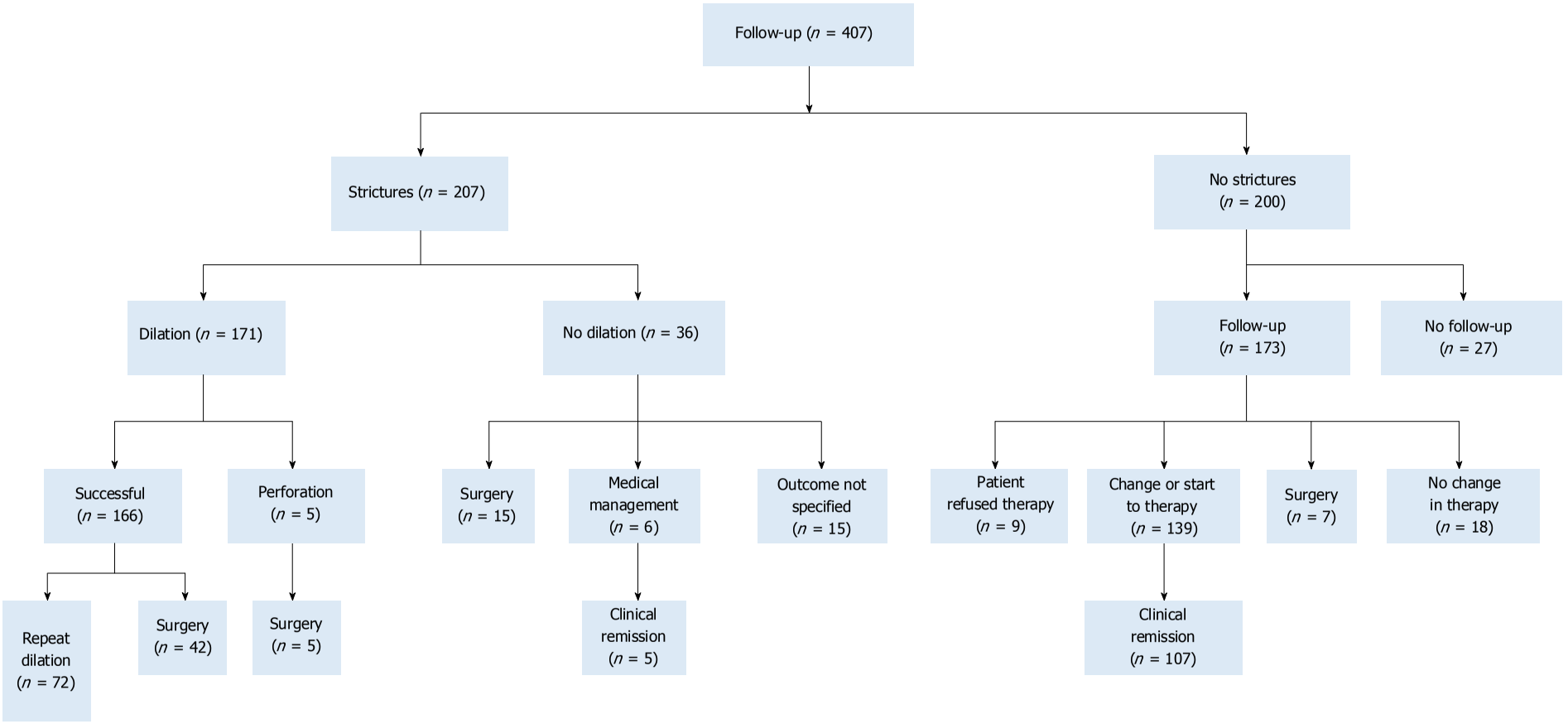

Figure 2 Outcome and impact of balloon-assisted enteroscopy in patients with follow-up.

complications.

COMMENTS

Background

Crohn's disease (CD) often affects the small bowel, but conventional upper and lower endoscopy are limited in their evaluation of small bowel disease activity or ability to perform interventions. Balloon assisted enteroscopy (BAE) allows for direct visualization and sampling of the small bowel, however its safety and role in CD remain to be established.

Research frontiers

This systematic review reveals that diagnostic BAE in CD has similar perforation rates as diagnostic BAE for all indications, suggesting that BAE is a safe way to evaluate small bowel CD. Further randomized controlled trials are warranted to

confirm these findings as well as to identify anatomical characteristics that are at highest risk for procedure related complications.

Innovations and breakthroughs

The research demonstrates that BAE has a similar perforation rate in diagnostic evaluation of small bowel CD *vs* other indications. This systematic review is the most up-to-date overview of this subject matter.

Applications

Diagnostic BAE in CD has a similar perforation rate as in other indications, thus it can be safely performed in diagnostic evaluation of small bowel CD.

Peer-review

The present manuscript is well written. The efficacy, safety and long-term prognosis of balloon dilation using BAE should be separately shown between patients with small bowel strictures and patients with anastomotic strictures. This should be analyzed in the manuscript.

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P- Reviewer: Kobayashi Y, Lakatos PL, Ogata H, Pinho R
S- Editor: Ma YJ **L- Editor**: A **E- Editor**: Zhang FF

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Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.3748/wjg.v22.i40.9012

World J Gastroenterol 2016 October 28; 22(40): 9012-9021
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

Efficacy of thioguanine treatment in inflammatory bowel disease: A systematic review

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Conflict-of-interest statement: The Department of Gastroenterology and Hepatology of the VU University Medical Center received an unrestricted research grant by TEVA Pharma BV outside of this submitted work.

Data sharing statement: Technical appendix, statistical code, and dataset available from the corresponding author at b.meijer1@vumc.nl. No additional data are available.

Manuscript source: Unsolicited manuscript

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Received: June 2, 2016
Peer-review started: June 3, 2016
First decision: July 12, 2016
Revised: July 20, 2016
Accepted: August 10, 2016
Article in press: August 10, 2016
Published online: October 28, 2016

Abstract

AIM

To critically assess the available literature regarding the efficacy of thioguanine treatment in inflammatory bowel disease (IBD) patients, irrespective of the (hepato-) toxicity profile.

METHODS

A systematic literature search of the MEDLINE database using PubMed was performed using the keywords "thioguanine", "6-TG", "thioguanine", "inflammatory bowel disease", "IBD", "Crohn's disease", "Ulcerative colitis" and "effectiveness" in order to identify relevant articles published in English starting from 2000. Reference lists of the included articles were cross-checked for missing articles. Reviewed manuscripts concerning the effectiveness of thioguanine treatment in IBD were reviewed by the authors and the data were extracted. Data were subsequently analyzed with descriptive statistics. Due to the lack of standardized outcomes, a formal meta-analysis was not performed.

RESULTS

A total of 11 applicable studies were found that involved the effectiveness of thioguanine therapy in IBD. Eight studies were conducted in a prospective

manner, in the remaining three studies, data was collected retrospectively. In total, 353 IBD-patients (225 patients with Crohn's disease, 119 with ulcerative colitis and nine with unclassified IBD) with prior azathioprine/mercaptopurine resistance and/or intolerance (*n* = 321) or *de novo* thioguanine administration (*n* = 32) were included for analysis, of which 228 (65%) had clinical improvement on thioguanine therapy, based on standard IBD questionnaires, biochemical parameters or global physician assessments. Short-term results were based on 268 treatment years (median follow-up 9 mo, range 3-22 mo) with a median daily dose of 20 mg (range 10-80 mg). Discontinuation, mostly due to adverse events, was reported in 72 patients (20%).

CONCLUSION

The efficacy of thioguanine therapy in IBD patients intolerant to conventional thiopurine therapy is observed in 65%, with short term adverse events in 20% of patients.

Key words: Thiopurines; Thioguanine; Inflammatory bowel disease; Crohn's disease; Ulcerative colitis

Core tip: Whereas conventional thiopurines are globally accepted as second-line treatment of inflammatory bowel disease (IBD) patients, almost half of these patients discontinues this treatment due to ineffectiveness or intolerance. In this systematic review, the efficacy of thioguanine treatment, a thiopurine with a less extensive and complex metabolism, is systematically assessed to determine if this drug is an alternative in the treatment of IBD patients intolerant or ineffective to azathioprine and/or mercaptopurine. We showed that up to 65% of patients benefit of a switch to thioguanine, thus preserving these patients from potentially more harmful and expensive treatment with biologicals.

Meijer B, Mulder CJJ, Peters GJ, van Bodegraven AA, de Boer NKH. Efficacy of thioguanine treatment in inflammatory bowel disease: A systematic review. *World J Gastroenterol* 2016; 22(40): 9012-9021 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i40/9012.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i40.9012

INTRODUCTION

Inflammatory bowel disease (IBD) encompasses both Crohn's disease (CD) and ulcerative colitis (UC) and forms a group of diseases characterized by idiopathic chronic inflammation of the gastrointestinal tract. It has worldwide a rising incidence[1]. IBD is characterized by recurrent periods of remission and relapse of disease and treatment of IBD is mainly aimed at induction and maintenance of remission[2,3]. Based on current step-up treatment guidelines (systemic) corticosteroids are the therapy of choice for inducing remission[2,3]. Thiopurines, such as azathioprine (AZA) or mercaptopurine (MP) may be added to corticosteroid therapy for maintaining remission and medication may be initiated during induction phase[4-6]. However, the use of thiopurines is limited, largely due to an extensive spectrum of adverse events witnessed in up to almost half of patients, especially within the first twelve months of treatment. Toxicity includes myelotoxicity, hepatotoxicity, pancreatitis and gastrointestinal (GI-) complaints[7,8].

Thiopurines were first described in the 1950s by Gertrude Elion and George Hitchings and comprised three chemical structures: 6-thioguanine (6-TG), MP and AZA[9]. AZA and MP are frequently being used as treatment for IBD, while TG is currently only used as experimental or rescue therapy. Metabolism of conventional thiopurines is complicated, leading to formation of several, toxic and non-toxic metabolites, whereas the metabolism of TG is less complicated and more directly leading towards the intended pharmacologically active products (Figure 1)[10-12]. Effects of thiopurines may be characterized by two groups of metabolites; methylated thiopurines [*e.g.,* 6-methylmercaptopurine (6-MMP)] and 6-thioguanine nucleotides (6-TGN). At relatively low dosages, as has been advocated in treatment of IBD, the anti-inflammatory effect of thiopurines is mainly mediated *via* inhibition of the small GTPase Rac1, leading to apoptosis of activated T-lymphocytes, whereas high dosages, as usual in oncological treatment, are associated with inhibition of DNA synthesis[13,14].

Based on these findings, it has been hypothesized that prescribing TG therapy instead of AZA/MP reduces generation of potentially toxic metabolites, such as the methylated metabolites, whilst it is primarily converted into the therapeutically aimed metabolite 6-TGN by bypassing several rate-limiting metabolic steps. The key reason for not introducing TG in the standard therapeutic armentarium of IBD appears to be the reported hepatotoxicity [*i.e.,* nodular regenerative hyperplasia (NRH) and sinusoidal obstruction syndrome] which has been described to be highly prevalent, especially with higher dosages of TG (median 40 mg/d)[15]. Interestingly, these findings were not corroborated in subsequent studies in which lower dosages of TG were used (20 mg/d), justifying additional research regarding relatively efficacy of low-dose TG therapy in IBD patients[16-20]. These data are depicted in Table 1. Other adverse events probably associated with TG use, as described in previous literature, are summarized in Table 2.

Two years ago, there were approximately 1500 TG users in The Netherlands, with no serious toxicity being reported[21]. Since TG has recently been registered as

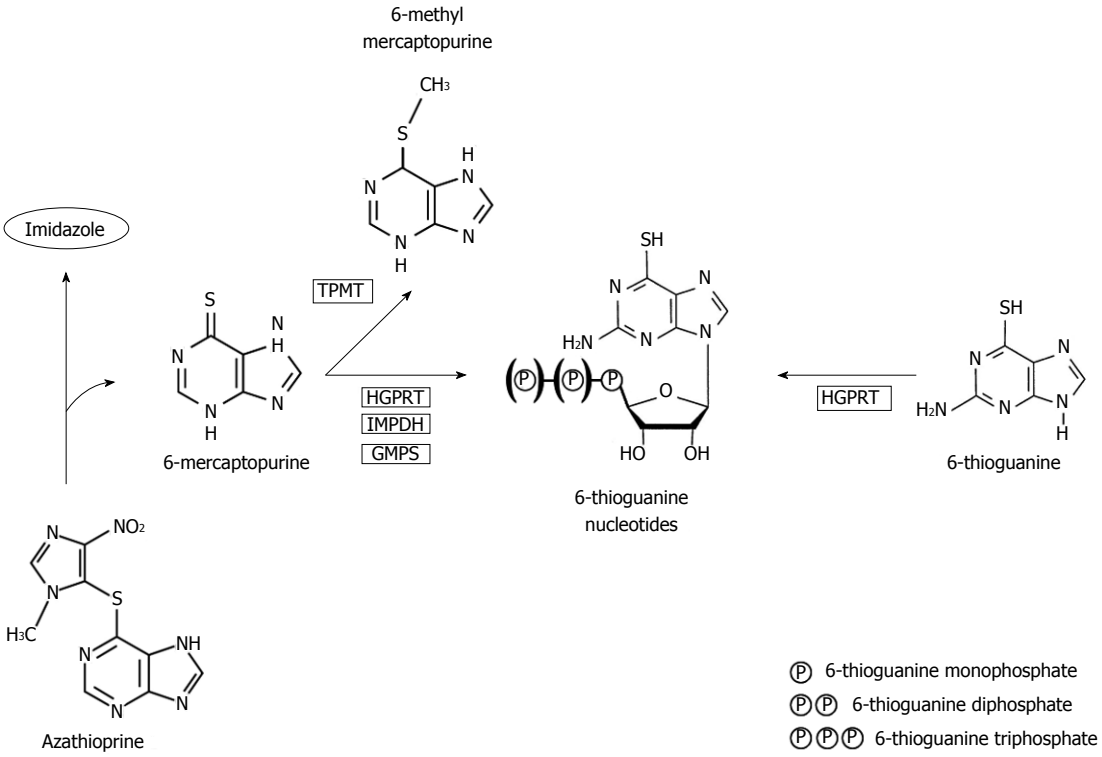

Figure 1 Simplified scheme of thiopurine metabolism. Azathioprine is non-enzymatically converted to 6-mercaptopurine by separating the imidazole-group. 6-Mercaptopurine is converted into 6-methylmercaptopurine (6-MMP) by thiopurine S-methyl transferase (TPMT) and into 6-thioguaninenucleotides (6-TGN) by an extensive enzymatic pathway using hypoxanthine-guanine phosphoribosyl transferase (HGPRT), inosine monophosphate dehydrogenase (IMPDH) and guanosine monophosphate synthetase (GMPS). 6-Thioguanine is converted directly into 6-TGN using HGPRT without producing the potentially toxic metabolite 6-MMP. Squared abbreviations display enzymatic conversions[46].

Table 1 Nodular regenerative hyperplasia of the liver during thioguanine therapy

Dosage of thioguanine	6-TGN level	Observed NRH	Ref.
About 20 mg per day (18-24 mg)	278 (68-492)	0% (0/12)	[20]
20 mg per day	564 ± 278	0% (0/28)	[18]
20 mg per day	802 (106-1092)	0% (0/13)	[47]
About 21 mg per day (0.3 mg/kg)	464 (65-1199)	6% (7/111)	[48]
40 mg per day	807 (105-2545)	0% (0/11)	[16]
About 40 mg per day (estimated)	1230 (530-2310)	62% (16/26)	[15]
40-80 mg per day	Unknown	36% (16/45)	[45]

6-TGN concentrations were calculated using the method described by Lennard *et al*[27]. Modified from Seinen *et al*[49]. 6-TGN: 6-thioguanine nucleotides concentration (as pmol/8 × 10^8 red blood cells), presented as medians with range or mean with standard deviation; NRH: Nodular regenerative hyperplasia.

certified treatment for IBD in The Netherlands, the question arises whether TG should be reconsidered as IBD treatment worldwide. The aim of this systematic review was to critically assess the available literature solely regarding the efficacy of TG treatment in IBD patients, irrespective of the alleged (hepato-)toxicity profile.

Table 2 Other adverse events associated with thioguanine use

Adverse event	Prevalence	Ref.
GI complaints	1%-17%	[16,17,19,20,37,38]
Myelosuppression[1]	1%-15%	[17,20,36,38]
General malaise	4%-22%	[17,38]
Allergic reaction	1%-6%	[17,33]
Other AE (*e.g.*, myalgia, alopecia)	1%-38%	[16,17,19,20,33,36-38]

[1]Variable definitions of myelosuppression were applied.

MATERIALS AND METHODS

This study was executed using the PRISMA guidelines[22]. We conducted a systematic literature search in the MEDLINE database using PubMed. We applied the following search strategy: ["Thioguanine"(Mesh) OR 6-TG (tiab) OR thioguanine (tiab) OR tioguanine (tiab)] AND ["Inflammatory Bowel Diseases"(Mesh) OR IBD (tiab) OR Crohn (tiab) OR Colitis (tiab)] AND (efficacy OR effectivity OR effectiveness).

Study selection

All studies were screened based on title and abstract. Full-text screening was performed in relevant studies

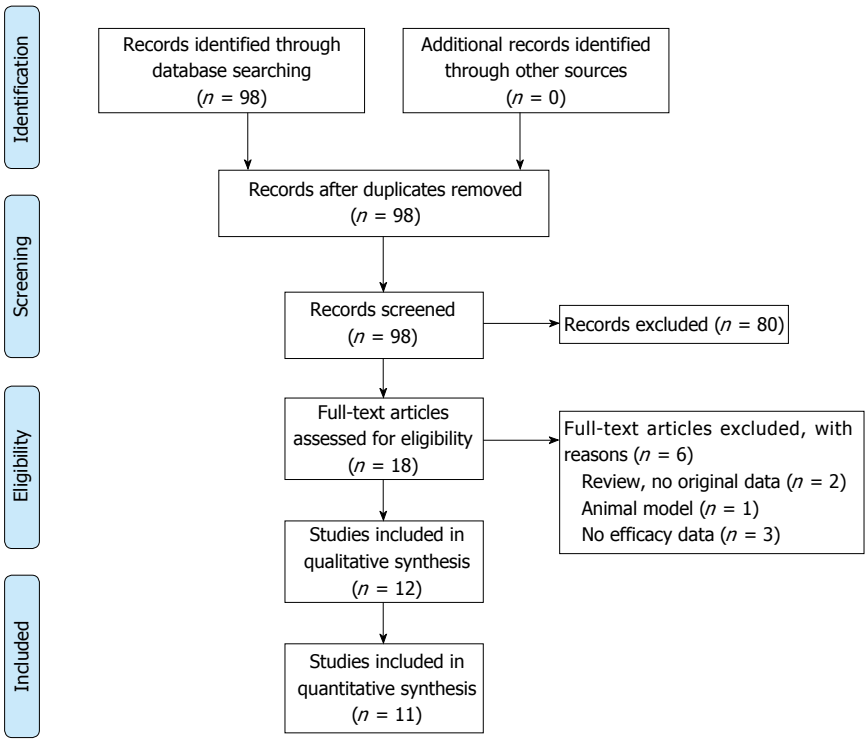

Figure 2 Flowchart of article selection procedure[22].

by the same authors (BM and NdB). The following inclusion criteria were met: patients diagnosed with IBD, TG therapy, efficacy as outcome, studies available in full-text in English or Dutch. Exclusion criteria were: in vitro studies, efficacy not identified as outcome, patients receiving TG therapy for other reasons than IBD, article not available in English or Dutch. Furthermore, all references of the included original papers were cross-checked to complete the search. All studies published from 2000 till 2016 were included in the systematic review. All studies with original study populations were included for analysis. Finally, authors of the included manuscripts were contacted in case of missing or unclear data or to identify additional studies.

Data extraction

If articles were eligible, we collected the following data from the original papers: study design, number of patients, patient characteristics, disease characteristics [*i.e.,* CD, UC or IBD unclassified (IBDu)], reason for initiation of TG, co-medication with corticosteroids, TG dose, duration of follow-up, efficacy of therapy, biochemical parameters [*i.e.*, C-reactive protein (CRP) and/or fecal calprotectin] and thiopurine drug metabolites (6-TGN and/or 6-MMP) during AZA/MP and TG treatment. Effectiveness of therapy was determined using endoscopic/clinical scoring scales [*i.e.,* Harvey-Bradshaw Index (HBI)[23], CD Activity Index (CDAI)[24], Colitis Activity Index (CAI)[25] or Simple Clinical Colitis Activity Index (SCCAI)[26]], as used in the different articles. Concentrations of 6-TGN were described using the method of Lennard *et al*[27] When the initial measurement was performed using the method described by Dervieux *et al*[28], this value was transposed into a calculated "Lennard value" as described by Shipkova *et al*[29].

RESULTS

The search strategy resulted in 98 papers. Most articles were excluded since these articles described measuring 6-TGN in patients treated with AZA or MP, instead of TG therapy. Thirteen were selected for full-text screening. One additional article was excluded because efficacy was not described.

Finally, twelve relevant articles were included (see Figure 2). Of these twelve articles, eleven studies comprised different study populations. One of the included papers is the extended follow-up period of another included paper, and was therefore not visualized in our primary overview (Table 3).

In the first study regarding TG-use[30] ten CD patients with therapeutic failure (*i.e.,* CDAI-scores above 150 and/or steroid-dependent disease) to AZA/MP therapy, combined with a preferential metabolite profile [defined as 6-TGN levels below 235 and 6-MMP levels above 6000 pmol/8 x 10^8 red blood cells (RBC)] were included. Nine patients were adults in whom TG was initiated at a dose of 40 mg/d, the pediatric patient (9 years old) was started on 20 mg/d. After 16 wk follow-up, eight patients were still using TG, in whom seven patients had a good clinical response, defined as a reduction in CDAI of at least 70 points or steroid reduction of at least 50%. 6-TGN levels

Table 3 Summary of included articles with most important study characteristics

Author	Ref.	Year	Score[1]	Number of patients	Number of IBD (CD/UC/IBDu)	Dose (med)	Follow-up (M)	Effective *n* (%)	Non-effective *n* (%)	Discontinuation *n* (%)	6-TGN (med)
Dubinsky	17	2001	Very low	10	10/0/0	40	4	7 (70)	1 (10)	2 (20)	1548[2]
Cheung	18	2003	Very low	15	13/1/1	40	3	12 (79)	1 (7)	2 (14)	N/A
Herrlinger	19	2003	Low	37	37/0/0	40	6	21 (57)	7 (19)	9 (24)	N/A
Bonaz	21	2003	Very low	49	49/0/0	20	12	38 (78)[3]	6 (12)	5 (10)	648[4]
Dubinsky	22	2003	Very low	21	14/7/0	20	9	14 (67)	3 (14)	4 (19)	1365[1]
Teml	23	2005	Low	20	0/14/6	20	6	11 (55)	3 (15)	6 (30)	816[4]
Qasim	24	2007	Very low	40	28/10/2	40	6	19 (48)	8 (20)	13 (32)	N/A
Ansari	12	2008	Low	30	30/0/0	40	6	18 (60)	5 (17)	7 (23)	807[4]
Almer	25	2009	Low	23	23/0/0	40	9	5 (22)	5 (22)	13 (56)	1155[2]
Asseldonk	16	2011	Low	46	0/46/0	20	22	37 (80)[3]	3 (7)	6 (13)	278[4]
Pavlidis	15	2014	Moderate	62	21/41/0	20	6	46 (78)	11 (14)	5 (8)	811[4]

[1]Grading based on GRADE guidelines[50,51]. [2]Median value in subgroup with clinical response to 6-thioguanine treatment; [3]Expected value; [4]Median value in total group, regardless of clinical response to treatment. IBD: Inflammatory bowel disease; CD: Crohn's disease; UC: Ulcerative colitis; IBDu: IBD unclassified; 6-TGN: 6-thioguanine nucleotides; N/A: Not applicable; Ref: Number in reference list; Dose: Median dosage of thioguanine therapy at initiation.

in these patients were median 1548 pmol/8 x 10^8 RBC (range 603-2073), with only one patient with a 6-TGN level below 1350. 6-MMP metabolites were undetectable in all patients. Biochemical parameters were not extensively reported. The two patients who discontinued TG treatment before week sixteen were excluded due to protocol violation, but were not reported to develop adverse events or to have an increase in IBD activity.

The next study[31] included fifteen IBD patients (13 CD/1 UC/1 IBDu) with either intolerance (*n* = 12) or inefficacy (*n* = 1) on AZA/MP therapy, as well as two thiopurine-naïve patients in which TG was started to "induce a quick therapeutic response". Fourteen patients were adults (range 23-65 years old) and one adolescent (17 years old). All patients were started on 40 mg TG. Based on global physician assessment (GPA), eleven patients (73%) had a good clinical response to TG therapy after a mean duration of only 3 wk. One additional patient had no decrease in CDAI but was able to successfully reduce prednisolone with > 50% and was classified as a partial response. There was a median follow-up of 16 wk (range 3-21 wk). Adverse events were described in four patients (27%) and were classified as mild. One patient had to discontinue TG treatment due to suspected pancreatitis (*i.e.,* slowly rising lipase concentration) and in three patients dosage was successfully reduced as diarrhea (*n* = 2) or leukopenia (*n* = 1) developed.

In a German study from 2003[32], 37 patients (22 with prior AZA intolerance, 15 thiopurine naive) with CD received 40 mg of TG daily. Dose was increased to 80 mg/d after 12 wk in non-responders and the effect was evaluated after a follow-up of 24 wk. Nine patients (24%) discontinued therapy before week 24 due to intolerance (*n* = 6), inefficacy (*n* = 2) or violation of protocol (*n* = 1). Of the remaining 28 patients, there were 21 patients (57%) with a clinical response, defined as a decrease in CDAI of > 70 points. Thirteen of these patients were in complete remission, of which twelve patients achieved this quiescent phase within four weeks of therapy. Twenty out of 27 patients (74%) on corticosteroids at initiation of TG were able to decrease steroids dosage with a median of 67% of initial steroid dose. CRP concentration was measured at baseline and at last follow-up, but there was no difference between these time points. In a second follow-up paper, the effect of maintenance treatment (total follow-up of one year) was evaluated[33]. Sixteen patients with continued use after six months of therapy were evaluated of which twelve were in remission with TG (*i.e.,* CDAI < 150) and four showed clinical response (defined as ΔCDAI > 70). Shortly after six months, two additional patients came into complete remission and patients in remission after six months maintained in remission after 12 mo of treatment. One patient with initial clinical response to TG relapsed and was switched to methotrexate therapy.

In another study from 2003[34] 49 patients with CD either intolerant for or refractory to AZA/MP therapy were included. All patients were adults and were started on 20 mg TG daily. Five patients (10%) out of 39 patients with prior intolerance to AZA/MP had to discontinue TG treatment due to (mild) adverse events within three weeks of therapy: nausea (*n* = 1), increase of hepatic enzymes (*n* = 2), vertigo (*n* = 1) and paresthesia (*n* = 1). After a median period of seven months, complete remission (defined as HBI below 3 and cessation of corticosteroids or infliximab) was achieved in 21 patients (43%). It was described that six patients (12%) relapsed on TG therapy. The remaining seventeen patients were not more extensively described in this study.

In a second study by Dubinsky *et al*[35], 21 patients were included with either CD or UC (14:7) who experienced a hypersensitivity reaction on conventional thiopurine therapy. All patients were adults. The dose of TG was not standardized and varied between 10 and 40 mg daily (median 20 mg/d). Four patients (19%) experienced a (mild) hypersensitivity reaction

on TG (two patients with gastrointestinal symptoms and two patients with flu-like illness). Of the remaining seventeen patients, fourteen (67%) improved on TG therapy after a median period of 9 mo, based on GPA. Two patients remained in remission and one patient had worsening of disease. 6-TGN concentrations were obtained in 14 of 17 patients and were all above 1100 pmol/8 × 10^8 RBC, irrespective of clinical response and not correlating with disease activity.

An Austrian research group[36] described fourteen UC patients and six IBDu patients with prior intolerance (n = 8) or inefficacy to previous AZA/MP treatment were reported. After a follow-up of 26 wk, eleven patients (55%) showed a therapeutic response (five with complete remission), defined as a CAI of 4 or lower. Three patients were classified as non-responders (15%), six patients discontinued treatment due to AE (n = 2) or non-compliance (n = 4, based on 6-TGN levels of below 250 pmol/8 × 10^8 RBC). Median 6-TGN level during therapy was 816 (range 279-2300), not correlating with response to therapy. Concentrations of CRP at follow-up did not differ from baseline concentrations.

An Irish population was included in another study[37] of 40 patients (28 CD, 10 UC and 2 IBDu) with prior inefficacy on AZA/MP in 21 patients or intolerance in 8 patients while *de novo* TG therapy was given in eleven patients. All patients were adults and started on 40 mg daily. After six months of therapy, TG had to be discontinued in thirteen patients (32%) due to AE, of which eight patients had hepatotoxicity (including thrombocytopenia, liver test abnormalities and splenomegaly). Nineteen patients (48%) had clinical benefit (*i.e.,* modified HBI or modified UC disease activity index below 4) of TG therapy and eight patients (20%) displayed no therapeutic response. Eleven patients were able to continue therapy over 1 year time period with therapeutic effect (complete remission in 10 patients). Furthermore, concentrations of CRP decreased during TG treatment when compared to baseline levels (P = 0.001).

Ansari *et al*[16] studied 30 CD patients with a median age of 34 years (range 12-57) treated with a median dose of 40 mg daily (range 20-60). All patients were either nonresponsive (n = 16) or intolerant (n = 14) to prior AZA treatment. After 6 mo there was a clinical response (*i.e.,* HBI < 5, in combination with successful withdrawal of steroids or infliximab) in eighteen patients (60%) and seven patients (23%) withdrew TG treatment due to AE. After six months another six patients developed AE leading to withdrawal of therapy. Eleven patients (37%) were able to continue therapy for a median period of 44 mo, leading to long-lasting remission. Five patients (17%) had no benefit from TG therapy. Median 6-TGN level was 807 pmol/8 × 10^8 RBC, there was no correlation between 6-TGN concentrations and clinical response.

In a Swedish study from 2009[38] 23 adult CD patients with prior thiopurine intolerance (n = 18) or resistance (n = 5) were treated with 40 mg (range 20-60 mg) TG once daily. After a median follow-up of 8 mo, thirteen patients (56%) had to discontinue treatment due to AE (n = 10) or unspecified safety concerns. Five patients (22%) had clinical response (defined as HBI < 5) on TG therapy, whilst five patients were non-responders. Median 6-TGN level in responding patients was 1155 (range 466-2488) pmol/8 × 10^8 RBC, however this result was not statistically different from non-responders [median 645 (range 551-1852), P = 0.73].

In a Dutch population[20] the sole focus was on UC patients and TG was introduced in a dose of approximately 0.3 mg/kg (median 20 mg/d, range 18-24) in 46 adult patients with either intolerance (n = 42) or refractoriness to AZA/MP. Within 6 mo, five patients had to discontinue treatment due to AE (n = 3) or were lost to follow-up. During follow-up, another three patients developed intolerance adding up to six patients (13%). Three patients experienced non-effectiveness on TG therapy and underwent colectomy. In the remaining 37 patients (80%), there was ongoing benefit and TG therapy was continued.

Finally, in a study of Pavlidis *et al*[19] performed in Australia and the United Kingdom, 62 adult patients (21 CD/41 UC) started on split-dose TG therapy of 20 mg once, twice or thrice daily after intolerance to conventional thiopurine therapy. After six months, 46 patients (78%) had a clinical response to TG therapy, defined as decrease in clinical activity scores (HBI $\leqslant$ 3 or SCCAI $\leqslant$ 2) and/or steroid use. Eleven patients (14%) did not benefit from treatment and had to undergo surgery. The remaining five patients discontinued treatment due to AE (n = 2) or were lost to follow-up. The median 6-TGN level was 811 pmol/8 × 10^8 RBC (range 340-2678) which did not correlate with disease activity.

Summary of treated patients

In summary, a total number of 353 (CD: 225/UC: 119/IBDu: 9) patients were treated with TG with a starting dose of 20 to 40 mg daily. The dosing was per individual adjusted to 10-80 mg/d, based on the development of adverse events or efficacy. Based on the median follow-up in the different studies, TG was administered for an estimated 268 treatment years. In 228 patients (65%), there was a benefit of TG therapy, defined as decrease in clinical disease symptom scales or the opportunity to cease or clinically significantly decrease corticosteroids without relapse of disease. No benefit of therapy was reported in 15% of patients, whereas 20% of the patients had to discontinue TG, mostly due to AE, comprising mainly gastrointestinal complaints, hypersensitivity reactions and elevated liver enzymes. In a subgroup analysis, 52% of CD patients and 62% of UC patients benefitted from TG therapy, whereas 11% of CD patients and 13% of UC patients had no benefit of therapy (Table 4).

Table 4 Summary of results of included articles in the total group, as well as different disease groups *n* (%)

Total number of patients	CD	UC	IBDu	Daily dose (mg)[1]	Treatment years[2]	Benefit[3]	No benefit	Discontinuation	
353	225 (64)	119 (34)	9 (2)	20 [10-80]	268	228 (65)	53 (15)	72 (20)	
								Discontinuation	Response unknown[4]
Crohn's disease (*n* = 225)					141	118 (52)	25 (11)	40 (18)	42 (19)
Ulcerative colitis (*n* = 119)					122	73 (62)	16 (13)	11 (9)	19 (16)

[1]Median start dose of thioguanine was 20 mg/d. This was adjusted based on symptoms and metabolite levels to doses of 10-80/d; [2]Treatment years are calculated based on sample size and median follow-up; [3]Defined as a clinically relevant decrease in disease activity scores by global physician assessment or when corticosteroids could be tapered or discontinued; [4]When results are not subdivided in disease entities (*i.e.*, total results are given, but not for CD/UC). CD: Crohn's disease; UC: Ulcerative colitis; IBDu: Inflammatory bowel disease unclassified.

DISCUSSION

In this study we systematically reviewed literature regarding the efficacy of TG treatment in IBD patients.

In 65% (range 22%-80%) of patients with active IBD treated with TG, mainly in patients failing prior conventional thiopurine therapy, clinical improvement was achieved. This was in line with recent reviews regarding efficacy of AZA or MP treatment in IBD: for maintenance therapy, efficacy of conventional thiopurine therapy was 73% and 50%, respectively. Induction therapy was effective in 30% and 51%, respectively[4,6,39,40].

Interestingly, most of the included patients experienced intolerance or inadequate response to previous conventional thiopurine therapy (*i.e.*, AZA/MP). Therefore the result of 65% is primarily based on patients with prior thiopurine exposure.

Eight studies (73%) were conducted in a prospective way, however no randomized trials have been performed to date. The study of Almer *et al*[38] is a relative negative outlier with only 22% response rate. This might be due to a small sample size (*n* = 23) in combination with a high number of discontinuation (*n* = 13). Three patients had to discontinue due to unspecified "safety reasons" and ten patients had adverse events leading to discontinuation. Five of them discontinued due to pain or gastrointestinal intolerance and two had mild hepatotoxicity with increasing bilirubin concentration or aminotransferase activity. On the contrary, two positive outliers were the studies of Bonaz *et al*[34] and van Asseldonk *et al*[20] with response rates of 78% and 80%, respectively. Interestingly, in these studies patients were started on 20 mg/d instead of 40 mg/d. This lower dosage might be the reason for better tolerability and could contribute to longer usage and subsequent higher efficacy.

The aim of this paper was to assess the effectiveness of TG treatment by a systematic review of available literature. Safety issues have extensively been reviewed (and nuanced) elsewhere (Table 1)[18,41-45]. However, since the majority of the included patients experienced adverse events on more conventional thiopurine derivatives, we compared the number of patients discontinuing treatment due to adverse events. Overall, 72 of 353 patients (20%) had to discontinue TG treatment, mainly due to adverse events. Interestingly, there seemed to be no increased risk of developing clinically overt non-cirrhotic portal hypertension due to NRH as compared to the study by Dubinsky *et al*[15] Other reasons for discontinuation were (unspecified) "safety reasons" or violation of applicable study protocol.

Concentrations of 6-TGN during TG treatment in none of the included studies (if available) showed a correlation with efficacy; its value in the management of TG therapy (therapeutic drug monitoring) can therefore not be extracted from the current series and, thus, warrant further analysis and study. However, patients with benefit of TG therapy showed median 6-TGN levels 1155, 1365 and 1548 pmol/8 × 10^8 RBC, respectively, in those studies in which this benefitting subgroup specifically was analyzed[15,35,38]. Based on these results, one may hypothesize that the therapeutic range of 6-TGN levels as proposed for conventional AZA/MP treatment (*i.e.*, > 230 pmol/8 × 10^8 RBC) is not applicable in patients treated with TG[46].

Several remarks have to be made about study design and patient population of the various included studies. All included studies are observational, open-label studies without control groups. A major part of discussion is the risk of bias in these kind of studies, especially publication bias. This type of bias is unavoidable in studies which are not previously registered in a trial registry, so the results in this review have to be interpret with this possible risk of bias taken into account. Furthermore, even though a larger part of the studies had a prospective design, no randomized trials are performed, yet, probably leading to confounding bias. Additionally, analyses in this paper were based on small patient groups (range 10-62) and effectiveness endpoints differed between the included studies, thwarting comparisons and robust conclusions.

Taken together, we critically reviewed the literature regarding effectiveness of TG treatment in IBD patients. Several small prospective trials showed encouraging results regarding therapeutic effect and

tolerability of TG in a population of IBD patients with reported intolerance or refractoriness to conventional thiopurine therapy with AZA or MP. These findings warrant randomized trials in IBD patients.

COMMENTS

Background

Conventional thiopurines play an important role in maintenance therapy of patients with inflammatory bowel disease (IBD). In a large proportion of IBD patients conventional thiopurine therapy fails, mainly due to adverse events. Thioguanine, another thiopurine derivative, has a less complicated metabolism and generates less potentially toxic metabolites and might serve as a rescue thiopurine.

Research frontiers

Thiopurines were first described by Gertrude Elion and George Hitchings in the 1950s. The conventional thiopurines (azathioprine and mercaptopurine) are frequently used in IBD treatment. The rediscovery of thioguanine led to the application of this drug as rescue treatment in IBD. In 2003, Dubinsky *et al* showed that over half of the patients treated with this drug (in high dose) developed nodular regenerative hyperplasia of the liver, leading to the immediate stop of thioguanine in IBD treatment. Over the years, these findings were not reproduced by other studies researching this topic.

Innovations and breakthroughs

Thioguanine has been successfully used in the treatment of IBD in various studies. Retrieved manuscripts (case series and observational cohort studies) concerning the effectiveness of thioguanine treatment in IBD were reviewed by the authors and the data were extracted.

Applications

This review suggests that thioguanine is an effective treatment option in IBD patients who experienced intolerance or ineffectiveness to conventional thiopurine treatment. This review may serve as fundament for prospective, randomized trials.

Terminology

Thioguanine is one of the three thiopurine derivatives used in the treatment of inflammatory bowel disease. Over the past decade, this drug has been 'rediscovered', but its use was limited due to alleged hepatotoxicity. Since March 2016, thioguanine has been registered as certified IBD treatment in The Netherlands.

Peer-review

This is a well-conceived and executed study in which the authors systematically reviewed literature regarding the efficacy of TG treatment in IBD patients. The study is an important one, was performed in an exemplary manner and is very well presented.

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P- Reviewer: Lakatos PL, Perse M, Tarnawski AS **S- Editor**: Qi Y
L- Editor: A **E- Editor**: Zhang FF

World Journal of Gastroenterology

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Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.3748/wjg.v22.i40.9022

World J Gastroenterol 2016 October 28; 22(40): 9022-9027
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

CASE REPORT

Safety and efficacy of self-expandable metallic stents in malignant small bowel obstructions

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Author contributions: Tsuboi A wrote the manuscript; Kuwai T assessed the patients; Nishimura T, Iio S and Mori T assisted in performing the diagnostic tests; Imagawa H and Yamaguchi T interpreted data; Yamaguchi A, Kouno H and Kohno H critically reviewed the manuscript.

Institutional review board statement: Ethical approval was not needed for this study, given the case series nature of the manuscript.

Informed consent statement: Informed consent was obtained from each of the patients.

Conflict-of-interest statement: There are no conflicts of interest to declare.

Manuscript source: Unsolicited manuscript

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Received: May 16, 2016
Peer-review started: May 17, 2016
First decision: July 12, 2016
Revised: August 25, 2016
Accepted: September 6, 2016
Article in press: September 6, 2016
Published online: October 28, 2016

Abstract

In this report, we present 3 cases of malignant small bowel obstruction, treated with palliative care using endoscopic self-expandable metallic stent (SEMS) placement, with the aim to identify the safety and efficacy of this procedure. Baseline patient characteristics, procedure methods, procedure time, technical and clinical success rates, complications, and patient outcomes were obtained. All 3 patients had pancreatic cancer with small bowel strictures. One patient received the SEMS using colonoscopy, while the other 2 patients received SEMS placement *via* double balloon endoscopy using the through-the-overtube technique. The median procedure time was 104 min. The technical and clinical success rates were 100%. Post-treatment, obstructive symptoms in all patients improved, and a low-residue diet could be tolerated. All stents remained within the patients until their deaths. The median overall survival time (stent patency time) was 76 d. SEMS placement is safe and effective as a palliative treatment for malignant small bowel obstruction.

Key words: Self-expandable metallic stents; Malignant small bowel obstructions; Endoscopy; Case report; Pancreatic cancer

Core tip: We present 3 cases of malignant small bowel

obstruction, treated with palliative care using endoscopic self-expandable metallic stent (SEMS) placement, and have identified that the procedure is safe and effective. Two patients were treated using the through-the-overtube technique, while the remaining case was the first case of SEMS placement in a malignant distal small bowel obstruction.

Tsuboi A, Kuwai T, Nishimura T, Iio S, Mori T, Imagawa H, Yamaguchi T, Yamaguchi A, Kouno H, Kohno H. Safety and efficacy of self-expandable metallic stents in malignant small bowel obstructions. *World J Gastroenterol* 2016; 22(40): 9022-9027 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i40/9022.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i40.9022

INTRODUCTION

Malignant small bowel obstructions are primarily treated with surgical intervention. However, palliative surgery is highly invasive in such patients, with poor prognosis; therefore, minimally invasive therapies, such as the endoscopic placement of self-expandable metallic stents (SEMS), have been considered. SEMS has been used successfully to palliate malignant gastrointestinal obstructions, and is widely reported to result in good clinical outcomes for colonic, esophageal, and gastric obstructions[1-4]. However, SEMS placement for malignant small bowel obstructions is little-known and also more challenging. A deep small bowel enteroscopy is limited, and three endoscopy systems are now available: [double balloon endoscopy (DBE), single balloon endoscopy, and spiral endoscopy[5-7]]; however, such endoscope systems do not have working channels large enough for the stent delivery systems to pass through. Therefore, the standard through-the-scope (TTS) technique for stent deployment could not be applied. To mitigate this limitation, we modified the standard over-the-guidewire (OTW) technique for stent deployment. In this report, we present 3 cases of malignant small bowel obstruction treated with palliative SEMS placement.

CASE REPORT

Of the 3 patients studied, 1 received stent deployment using the standard TTS technique *via* colonoscopy (CF-H260AI, Olympus, Tokyo, Japan); SEMS placement in the other 2 patients was achieved using the through-the-overtube (TTO) technique *via* DBE (EN450T5/W, FUJIFILM, Tokyo, Japan). The TTO technique is a modified version of the OTW technique. First, the endoscope with the overtube (TS13140, FUJIFILM, Tokyo, Japan), without its balloon tip, was advanced towards the stricture, and a 0.035-inch guidewire (Jagwire, Boston Scientific Corp., Natick, MA, United States) was passed through the stricture. Subsequently, the guidewire and the overtube were left in place, and the endoscope was removed. Finally, the overtube was utilized as a large channel to advance the stent through the stricture over the guidewire under fluoroscopic guidance. All 3 cases were performed by a single expert endoscopist with experience in over 20 cases of colon stenting.

Case 1

A 60-year-old woman was admitted to our hospital with abdominal pain due to terminal ileum obstruction because of peritoneal dissemination of pancreatic cancer. Although her symptoms improved after insertion of an ileus tube, they recurred following commencement of oral intake. Consequently, the decision was made to attempt SEMS placement as a palliative therapy. A colonoscope was advanced to the stricture, and the standard TTS technique for stenting with a 10 cm × 20 mm uncovered SEMS (Niti-S biliary stent, TaeWoong Medical, Seoul, South Korea) (Figure 1) was used. The patient was discharged 24 d after stenting, and died 109 d after stenting.

Case 2

An 87-year-old woman was admitted to our hospital with anorexia, vomiting, and weight loss. An abdominal computed tomography (CT) scan revealed cancer in the head of the pancreas, a metastatic hepatic tumor, and expansion of the stomach and duodenum. We concluded that the obstruction of the distal duodenum/angle of Treitz was secondary to pancreatic cancer invasion. We attempted advancement of a colonoscope (CF-H260AZI, Olympus, Tokyo, Japan) to the stricture, but could not reach the region as it was too deep, and the endoscope position was tortuous. Subsequently, DBE endoscopy was performed, and access was achieved with a stable endoscope position. Unfortunately, we could not use the TTS technique for stent deployment given the position of the stricture. Therefore, we decided to employ the TTO technique for stenting. An endoscope loaded with a balloon overtube was advanced into the stricture for a trans-oral approach. While the guidewire was left in place beyond the stricture, the endoscope was removed, leaving the overtube in place. A 10 cm × 22 mm stent (Niti-S D pyloric/duodenal stent, TaeWoong Medical, Seoul, South Korea) was advanced using the OTW technique through the overtube, and was deployed successfully (Figure 2). The patient was discharged 15 d after the procedure and died 76 d after stenting.

Case 3

A 69-year-old woman with Stage IV pancreatic cancer who was receiving chemotherapy was admitted due to abdominal distension and vomiting. Abdominal CT revealed an intestinal stricture secondary to peritoneal dissemination. She was initially treated with ileus tube insertion for the obstruction (due to recurrence), but requested palliative SEMS placement. As we

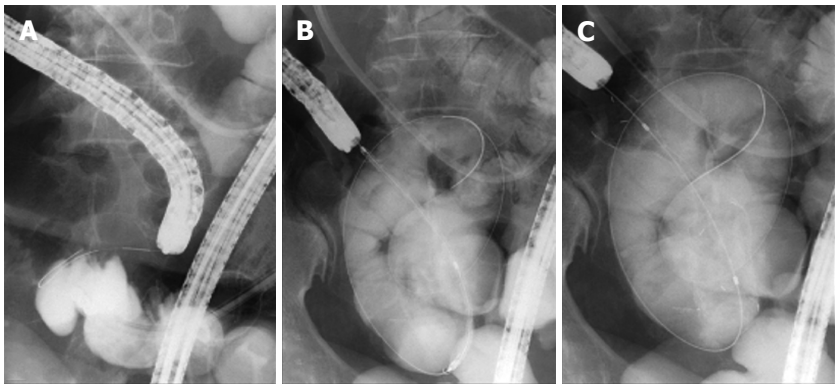

Figure 1 Self-expandable metallic stent deployment using the standard through-the-scope technique under fluoroscopic guidance. A: The scope was advanced to the stricture, and a standard guidewire was passed through the stricture; B: The stent delivery system was advanced through the scope across the stricture; C: The stent can be seen successfully deployed across the stricture.

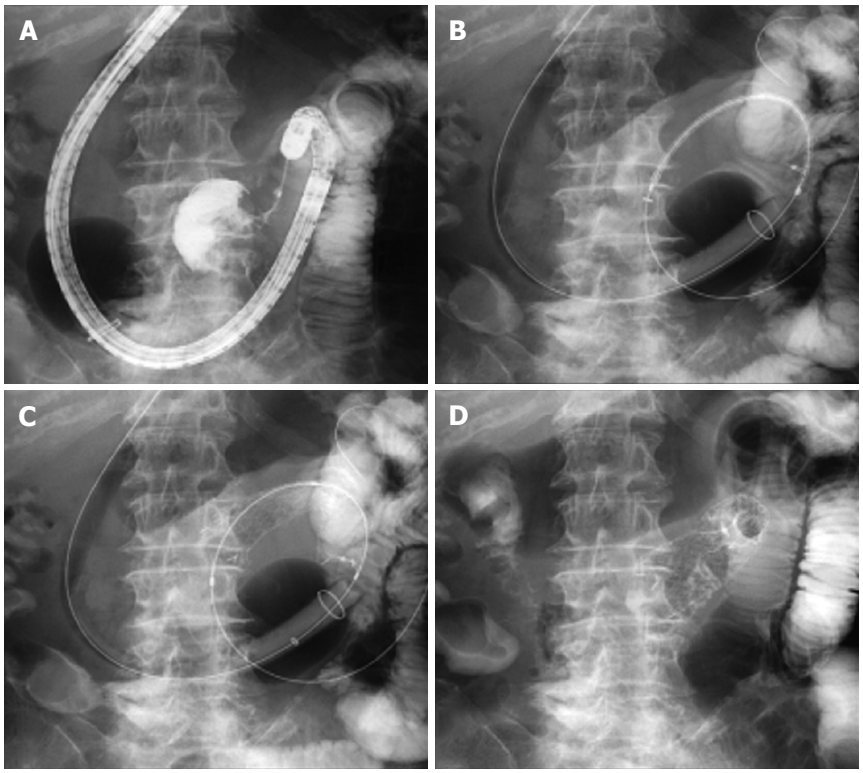

Figure 2 Self-expandable metallic stent deployment using the through-the-overtube technique under fluoroscopic guidance. A: The DBE was advanced to the stricture, and a standard guidewire passed through the stricture; B: The stent delivery system was advanced over the wire through the overtube; C: The stent deployed across the stricture; D: The overtube was withdrawn.

were able to reach the stricture with DBE, we decided to place the SEMS using the TTO technique. The endoscope and overtube were advanced to the stricture *via* the trans-anal approach. The guidewire (Wrangler, PIOLAX medical devices Inc., Kanagawa, Japan) and overtube were left in place while the endoscope was removed. An 8 cm × 18 mm stent (Niti-S D colonic stent, TaeWoong Medical, Seoul, South Korea) was advanced through the overtube, and deployed successfully (Figure 3). The patient was discharged on day 12 after the procedure and died of her primary cancer 29 d after stenting.

All 3 patients in our study tolerated clear fluids the day after stenting, followed by a low residue diet. They were all discharged from the hospital at variable times with no major complications following SEMS placement (Table 1; summary of cases). All stents remained patent until patient death. The technical and clinical success rates were 100%.

DISCUSSION

Malignant small bowel obstructions are typically caused by primary small bowel malignant tumors, local invasion

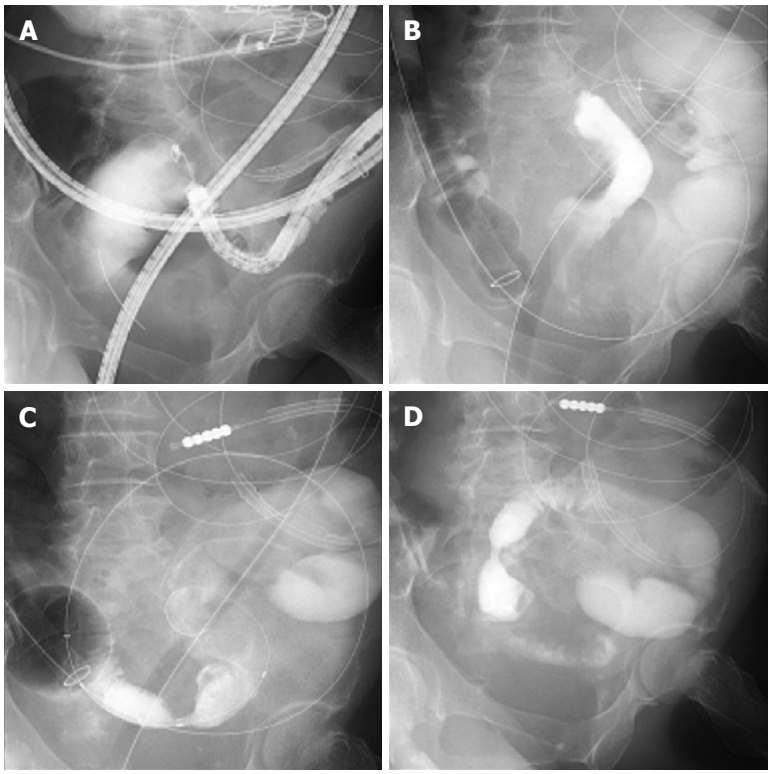

Figure 3 Self-expandable metallic stent deployment using the through-the-overtube technique *via* a trans-anal approach under fluoroscopic guidance. A: The DBE was advanced to the stricture, and a guidewire passed through the stricture; B: The guidewire and overtube were left in place and the endoscope was removed; C: The stent delivery system was advanced over the wire through the overtube, and the stent deployed across the stricture; D: The overtube was withdrawn.

Table 1 Summary of cases

Age/sex	Tumor	Stricture location	Scope	Stent delivery	Stricture length	Type of stent	Procedural time	Stent patency time	Time to oral intake after stent placement
60/F	Pancreatic cancer Peritoneal dissemination	Terminal ileum	Olympus CF-H260A	TTS	40 mm	Niti-S 20 mm × 10 cm	132 min	109 d	5 d
87/F	Pancreatic cancer	Proximal jejunum	FUJIFILM EN-450T5/W (trans-oral)	TTO	30 mm	Niti-S 22 mm × 10 cm	46 min	76 d	2 d
60/F	Pancreatic cancer Peritoneal dissemination	Distal ileum	FUJIFILM EN-450T5/W (trans-anal)	TTO	20 mm	Niti-S 18 mm × 8 cm	104 min	29 d	2 d

TTS: Through-the-scope; TTO: Through-the-overtube.

of extrinsic malignant tumors, or metastasis[8]. Although surgical interventions such as gastroenteric bypass or ileostomy are considered the primary treatment for patients with malignant small bowel obstructions, they are not routinely performed in view of poor prognoses. Recently, SEMS placement has been used to treat malignant, non-small bowel gastrointestinal obstructions. Compared with surgery, SEMS placement is much less invasive. Good clinical outcomes have been reported in esophageal, gastroduodenal, and colorectal malignant obstructions[1,9]. While the efficacy and safety of palliative SEMS placement in these types of malignant obstructions are well-established, it remains largely unknown how such parameters measure in malignant small bowel obstructions distal to the ligament of Treitz. In this study, the technical and clinical success rates were 100%, with no major complications observed. Therefore, we propose that SEMS placement for malignant small bowel obstruction is equally effective and safe.

SEMS placement for malignant small bowel obstruction is challenging given the difficulty in accessing the site. Jeurnink *et al*[10] reported that enteral SEMS placement could be effectively and safely performed

for malignant obstructions of the distal duodenum or proximal jejunum with colonoscopy. SEMS placement *via* colonoscopy is preferable for treatment of these lesions, considering the scope length and working channel, which is large enough for the standard TTS technique. In case 1, we used a colonoscope to deploy the SEMS with the TTS technique, as the scope could be advanced to the malignant stricture of the terminal ileum; the stenting was performed successfully.

Few reports on SEMS placement for malignant small bowel obstructions have been published. Lee *et al*[11] reported on 19 patients with malignant small bowel obstructions who underwent SEMS insertion. In these patients, SEMS placement was performed with the withdrawal-reinsertion technique using DBE. In their report, the technical and clinical success rates were 95% and 84%, respectively, and no major complications were observed during the procedures. According to the report, SEMS placement appeared to be effective for palliation of malignant small bowel obstructions. However, patients with malignant distal small bowel obstructions were excluded from the report; therefore, the efficacy and safety of SEMS placement in these regions was largely unknown.

As current enteroscopy systems do not possess working channels large enough for stent delivery systems to pass through, SEMS placement utilizing the TTS technique with an enteroscope is not possible. Therefore, the TTO technique was developed by modifying the OTW technique for SEMS placement in malignant distal small bowel obstructions using DBE. Ross *et al*[5] reported a case of malignant distal duodenal obstruction treated with SEMS placement using DBE with the TTO technique, similar to case 2 of our study. Lennon *et al*[6] reported a similar technique using SE. The key to this technique is the ability to reach the stricture and lock the overtube in position at the location; this provides a sheath through which the stent could easily pass the stricture and be deployed. Although this technique can potentially treat deeper malignant small bowel obstructions, the few case reports that are available have only used this technique in the distal duodenum, proximal jejunum, or surgically-reconstructed intestines[5,6,11-14]. To the best of our knowledge, case 3 of our study is the first case of SEMS placement in a malignant distal small bowel obstruction.

Shimatani *et al*[15] recently reported on SEMS placement for malignant afferent-loop obstruction using the TTS technique with a new short-type DBE (EI-580 BT; Fujifilm, Tokyo, Japan). As the new short-type DBE has a 3.2-mm working channel, the 9 Fr SEMS delivery system can be used with the TTS technique. We believe that a long-type DBE (also with a 3.2-mm working channel) will be developed in the near future. This will allow treatment of deeper malignant small bowel obstructions with SEMS placement using the TTS technique.

In conclusion, our study revealed that palliative SEMS placement is safe and effective in malignant small bowel obstructions. However, given our small sample size, further studies are warranted. Nevertheless, we believe that SEMS placement will play a significant role in the primary treatment of malignant small bowel obstructions in the near future, with further development of endoscopy and SEMS delivery systems.

ACKNOWLEDGMENTS

The authors thank Naoko Matsumoto for assistance in collecting data and for office procedures.

COMMENTS

Case characteristics

Three patients (a 60-year-old woman, an 87-year-old woman, and a 69-year-old woman) presented with small bowel obstruction due to pancreatic cancer.

Clinical diagnosis

An abdominal computed tomography (CT) scan revealed the clinical diagnoses in all cases.

Imaging diagnosis

Abdominal CT showed small bowel obstruction because of pancreatic cancer.

Treatment

Endoscopic self-expandable metallic stents (SEMS) were placed in each patient.

Related reports

Only a few reports regarding SEMS placement for malignant small bowel obstructions have been published. Notably, case 3 in this study may be the first reported case of SEMS placement in a malignant distal small bowel obstruction.

Term explanation

through-the-scope was defined as tube-through-the-scope, over-the-guidewire was defined as over-the-guidewire, and through-the-overtube was defined as through-the-overtube.

Experiences and lessons

The authors present 3 cases of malignant small bowel obstruction that received palliative SEMS placement safely and effectively. The SEMS placement will play a significant role in the primary treatment of malignant small bowel obstruction in the near future, with further development of endoscopy and SEMS delivery systems.

Peer-review

The authors presented 3 cases of malignant small bowel obstruction treated with palliative care by endoscopic SEMS placement and revealed that the procedure is safe and effective. The findings will be of interest to the readership.

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P- Reviewer: Castro FJ, Lakatos PL, Manguso F, Sadik R
S- Editor: Qi Y **L- Editor**: A **E- Editor**: Zhang FF

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DOI: 10.3748/wjg.v22.i40.9028

World J Gastroenterol 2016 October 28; 22(40): 9028-9034
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

CASE REPORT

Two cases of adenocarcinoma occurring in sporadic fundic gland polyps observed by magnifying endoscopy with narrow band imaging

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Author contributions: Togo K and Ueo T designed and wrote the paper; Togo K, Ueo T and Honda H analyzed the data and treated the patient; Yonemasu H, Tanabe H and Iwashita A made pathological diagnosis; Ishida T and Murakami K made critical revision of the article for important intellectual content; Yao K advised for endoscopic findings.

Institutional review board statement: Oita Red Cross Hospital Institutional Review Board dose not require approval for case reports.

Informed consent statement: The patients involved in this case report authorized the disclosure of their protected health information for academic purposes.

Conflict-of-interest statement: All authors indicate that they have no financial interests or potential conflicts of interest to declare.

Manuscript source: Invited manuscript

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Received: July 11, 2016
Peer-review started: July 13, 2016
First decision: August 8, 2016
Revised: August 22, 2016
Accepted: September 12, 2016
Article in press: September 12, 2016
Published online: October 28, 2016

Abstract

Gastric fundic gland polyps (FGPs) are common non-adenomatous gastric polyps arising from normal fundic mucosa without *Helicobacter pylori* (*H. pylori*) infection. Although systemic FGPs associated with familial adenomatous polyposis (FAP) often have dysplasia, there are few reports of dysplasia occurring in sporadic FGPs, especially when detected by magnifying endoscopy with narrow band imaging (ME-NBI). We experienced two cases of adenocarcinoma occurring in sporadic FGPs, and their ME-NBI findings were very useful for differentiating FGP with cancer from non-dysplastic FGP. A 68-year-old man and a 63-year-old woman were referred to our institution for medical checkup. *H. pylori* was negative in both patients. Endoscopic examination revealed a small reddish

polypoid lesion on the anterior wall of the upper gastric body and several FGPs. ME-NBI showed an irregular microvascular architecture composed of closed loop- or open loop-type vascular components, plus an irregular microsurface structure composed of oval-type surface components which was different from that of FGPs. FAP was denied because of the absence of colon polyps and no familial history of FAP. Pathological diagnosis was adenocarcinoma occurring in sporadic FGP.

Key words: Sporadic type; Adenocarcinoma; Magnifying endoscopy with narrow band imaging; Adenocarcinoma without *Helicobacter pylori* infection; Fundic gland polyp

Core tip: Gastric fundic gland polyps (FGPs) are common non-adenomatous gastric polyps arising from normal fundic mucosa without *Helicobacter pylori* infection. Although systemic FGPs associated with familial adenomatous polyposis often have dysplasia, there are few reports of dysplasia occurring in sporadic FGPs, especially when detected by magnifying endoscopy with narrow band imaging (ME-NBI). We experienced two cases of adenocarcinoma occurring in sporadic FGPs. ME-NBI showed an irregular microvascular architecture plus an irregular microsurface structure which was different from that of FGPs. ME-NBI findings were very useful for differentiating FGP with cancer from non-dysplastic FGP.

Togo K, Ueo T, Yonemasu H, Honda H, Ishida T, Tanabe H, Yao K, Iwashita A, Murakami K. Two cases of adenocarcinoma occurring in sporadic fundic gland polyps observed by magnifying endoscopy with narrow band imaging. *World J Gastroenterol* 2016; 22(40): 9028-9034 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i40/9028.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i40.9028

INTRODUCTION

Gastric fundic gland polyps (FGPs) are the most common non-adenomatous gastric polyps composed of cystically dilated fundic glands beneath a normal gastric foveolar epithelium without *Helicobacter pylori* (*H. pylori*) infection and atrophic gastritis[1]. FGPs were initially recognized as one of the gastric lesions in patients with familial adenomatous polyposis (FAP). In such systemic FGPs, foveolar dysplasia and rarely invasive gastric adenocarcinoma have been reported[2,3]. In contrast to FGPs without FAP, namely "sporadic FGPs", it is extremely rare to encounter dysplasia, even though it is frequently encountered in sporadic FGPs in daily clinical practice[2,4-6].

Despite the strong link between *H. pylori* infection and gastric cancer, the new entity "adenocarcinoma of fundic gland type" has been increasingly reported as a representative neoplasia with no *H. pylori* infection[7]. An increased risk of FGPs has been reported with long term use of proton pump inhibitors (PPIs) therapy in *H. pylori*-negative patients with symptomatic gastroesophageal reflux disease[8]. Although it is rare, endoscopists must be vigilant for dysplasia from FGPs as one of representing neoplasias with no *H. pylori* infection. However, the endoscopic finding of FGPs with dysplasia, especially sporadic cases, has not been described. Furthermore, there has been no report of findings using magnifying endoscopy with narrow band imaging (ME-NBI).

We experienced two cases of adenocarcinoma occurring in sporadic FGP, and their ME-NBI findings were very useful for differentiating dysplastic from non-dysplastic FGP.

CASE REPORT

Case 1

A 68-year-old man was referred to our institution for medical checkup. He had no medical history of PPIs use and no family history of FAP. *H. pylori* was negative by urea breath test and antibody in blood serum. Upper endoscopic examination revealed a reddish polypoid lesion of approximately 5 mm in size adjacent to an isochromatic small polyp that was thought to be FGP on the anterior wall of the upper gastric body (Figure 1A). In addition to these two polypoid lesions, there were several FGPs in non-atrophic background mucosa. ME-NBI of the reddish polypoid lesion showed an irregular microvascular (MV) architecture composed of closed loop- or open loop-type vascular components, with an irregular microsurface structure (MS) composed of oval-type surface components (vessels within epithelium pattern; Figure 1B). In contrast, ME-NBI of the adjacent isochromatic polyp showed regularly arranged round gastric pits with regularly arranged honeycomb-like microvessels (epithelium within vessels pattern; Figure 1C). Biopsy specimen from the reddish polypoid lesion was suspicious for gastric adenocarcinoma of differentiated type. From these findings, we suspected that the reddish polypoid lesion was an intramucosal adenocarcinoma of differentiated type, whereas the adjacent isochromatic polyp was a non-dysplastic FGP.

After obtaining informed consent, we resected these two polypoid lesions by endoscopic submucosal dissection (ESD). Histological examination of the en bloc specimen showed that the reddish polypoid lesion was very well-differentiated adenocarcinoma (Figure 2A and B). Irregularly branching tumor glands with atypical nuclei were found in the surface part of the lesion, and proliferation of fundic glands was observed with some cystic dilatation at the basal part of the lesion (Figure 2B and C). Immunohistochemical study

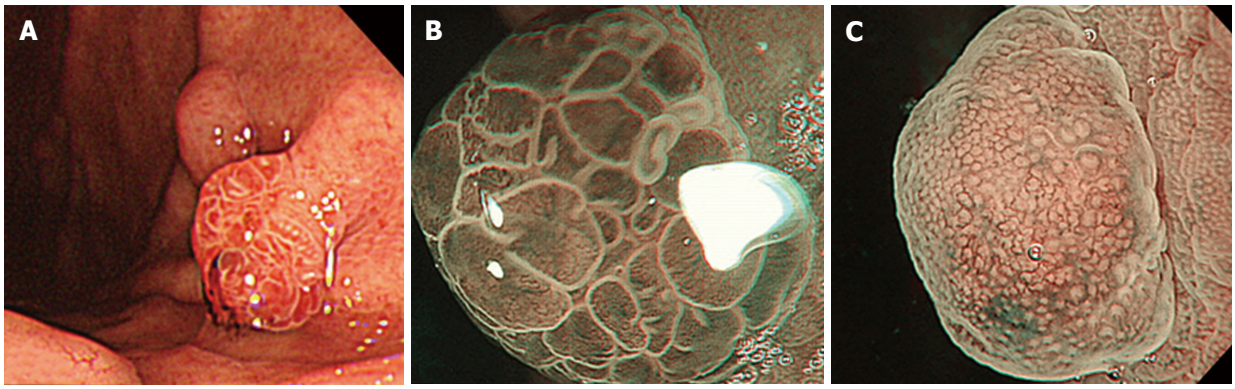

Figure 1 Conventional endoscopy in Case 1. Reveals a reddish polypoid lesion adjacent to an isochromatic small polyp on the anterior wall of the upper gastric body (A). ME-NBI of the reddish polypoid lesion shows an irregular microvascular architecture composed of closed loop- or open loop-type vascular components, plus irregular microsurface structure composed of oval-type surface components with demarcation line (vessels within epithelium pattern; B); ME-NBI of the adjacent isochromatic polyp shows regularly arranged round gastric pits within regularly arranged honeycomb-like microvessels with demarcation line (epithelium within vessels pattern; C).

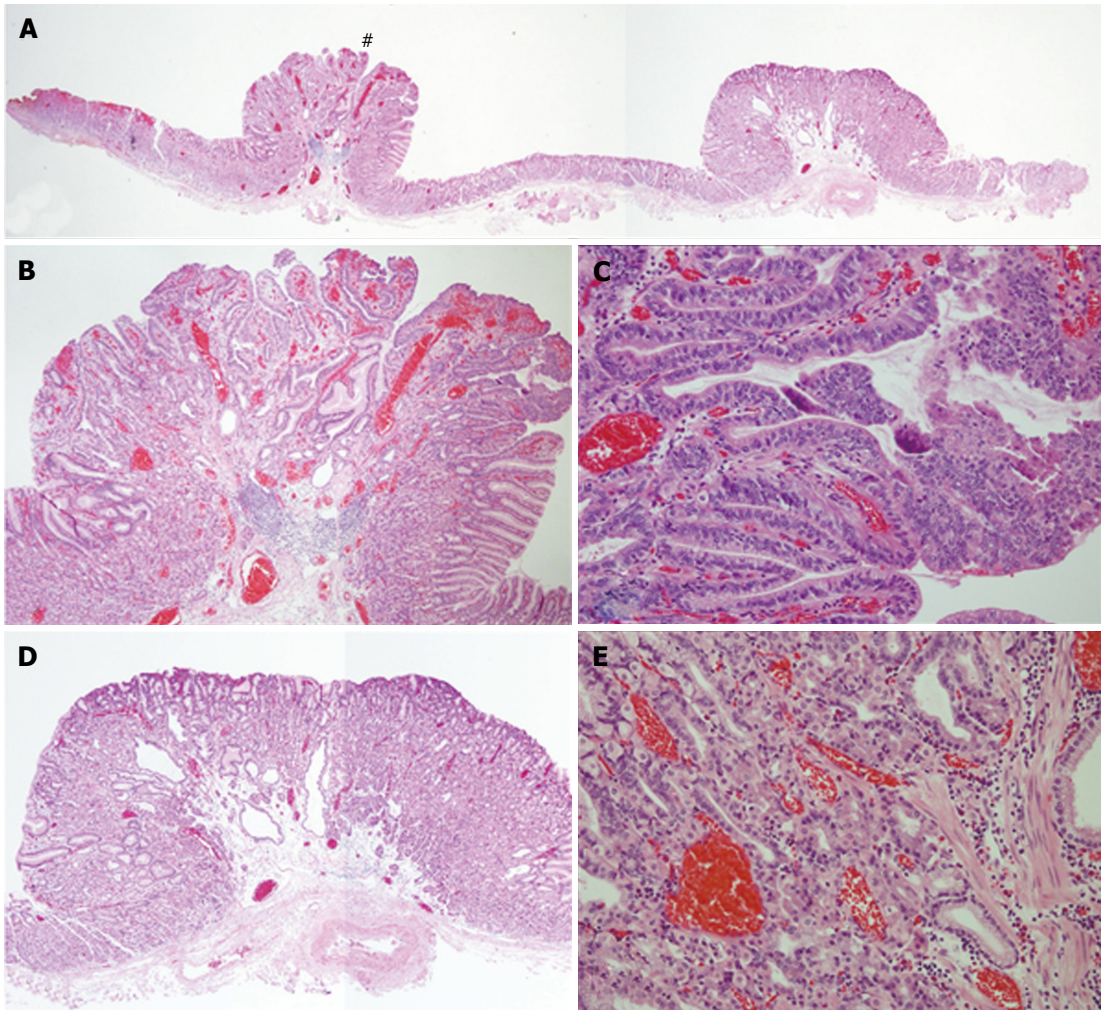

Figure 2 Histological examination of the endoscopic submucosal dissection specimen from Case 1. Shows that the left side lesion (#) is consistent with reddish polypoid and the right side lesion is consistent with isochromatic small polyp (A). High magnification of the left side lesion (#) shows irregularly branching tumor glands with atypical nuclei at the surface part of the lesion, and proliferation of fundic glands with some cystic dilatation at the basal part of the lesion, which was diagnosed as very well-differentiated adenocarcinoma occurring in FGP (B and C). High magnification of the right side lesion shows proliferation of fundic glands with some cystic dilatation, which was diagnosed as FGP without dysplasia (D and E). FGP: Gastric fundic gland polyp.

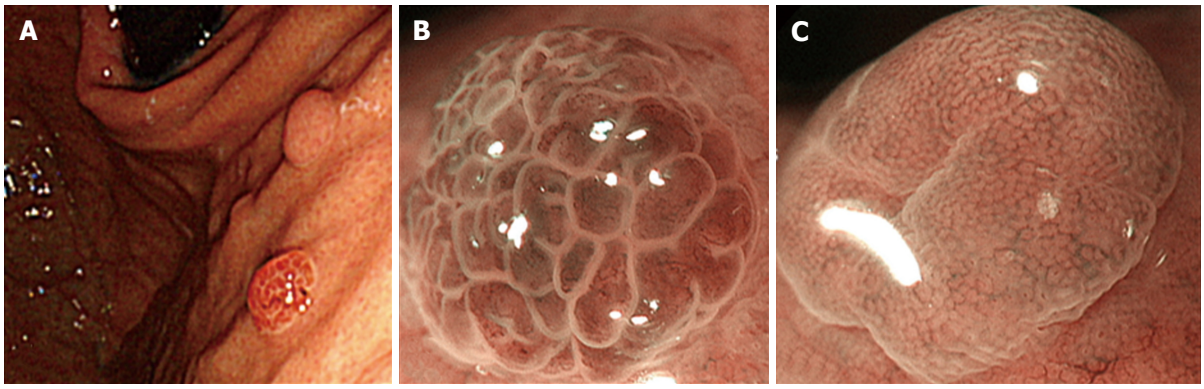

Figure 3 Conventional endoscopy in Case 2. Reveals a reddish polypoid lesion adjacent to an isochromatic small polyp on the anterior wall of the upper gastric body (A). ME-NBI of the reddish polypoid lesion shows an irregular microvascular architecture composed of closed loop- or open loop-type vascular components, plus irregular microsurface structure composed of oval-type surface components with demarcation line (vessels within epithelium pattern; B). ME-NBI of the adjacent isochromatic polyp shows regularly arranged round gastric pits within regularly arranged honeycomb-like microvessels with demarcation line (epithelium within vessels pattern; C).

showed that the tumor cells were positive for MUC5AC and negative for MUC6 pepsinogen-I, H, K-ATPase, CD10, MUC2 and p53. Ki67 was diffusely present in tumor cells. Beta-catenin was positive on the cell membrane of both neoplastic and non-neoplastic cells. The final pathological diagnosis was very well-differentiated adenocarcinoma occurring in FGP (4 mm × 4 mm in size, tub1, pT1a (M), ly0, v0, pHM0, pVM0). In contrast, histological examination of the adjacent isochromatic polyp showed proliferation of fundic glands with some cystic dilatation, and was diagnosed as FGP without dysplasia (Figure 2A, D and E). FAP was denied because of the absence of colon polyps and no familial history of FAP.

Case 2

A 63-year-old woman was referred to our institution for management of a newly diagnosed gastric tumor. She had no medical history of PPIs use and no family history. *H. pylori* was negative by antibody in blood serum and antigen in stool. Upper endoscopic examination revealed a reddish polypoid lesion approximately 3 mm in size adjacent to an isochromatic small polyp that was thought to be FGP on the anterior wall of the upper gastric body (Figure 3A). There were many additional FGPs in non-atrophic background mucosa as well. ME-NBI of the reddish polypoid lesion and adjacent isochromatic polyp were almost the same as described for Case 1. Briefly, the reddish polypoid lesion showed an irregular MV pattern and irregular MS pattern with demarcation (vessels within epithelium pattern; Figure 3B), while the adjacent isochromatic polyp showed a regular MV pattern and regular MS pattern with demarcation (epithelium within vessels pattern; Figure 3C). Although biopsy specimen from the reddish polypoid lesion was suspicious for adenoma, we suspected that the reddish polypoid lesion was an adenocarcinoma occurring in FGP as we experienced in Case 1, whereas the adjacent isochromatic polyp was non-dysplastic FGP. After obtaining informed consent, we resected these two polypoid lesions by ESD. Histological examination of the en bloc specimen showed that the reddish polypoid lesion was very well-differentiated adenocarcinoma (Figure 4A and B). Irregularly branching tumor glands with atypical nuclei were found at the surface part of the lesion, and proliferation of fundic glands with some cystic dilatation were observed at the basal part of the lesion (Figure 4B and C). Immunohistochemical study showed that the tumor cells were positive for MUC5AC and negative for MUC6, CD10, MUC2 and p53. Ki67 was diffusely distributed in tumor cells. Beta-catenin was positive on the cell membrane of both neoplastic and non-neoplastic cells. The final pathological diagnosis was very well-differentiated adenocarcinoma occurring in FGP [3 mm × 3 mm in size, tub1, pT1a (M), ly0, v0, pHM0, pVM0]. In contrast, histological examination of the adjacent isochromatic polyp showed proliferation of fundic glands with some cystic dilatation, and was diagnosed FGP without dysplasia (Figure 4A, D and E). FAP was denied because of the absence of colon polyps and no familial history of FAP.

DISCUSSION

FGPs are the most common types of gastric polyps. Stolte *et al*[9] reported that FGPs were found in 47.0% of all types of gastric polyps[9]. Genta *et al*[10] reported that FGPs were found in up to 5.9% of adults undergoing upper endoscopic examination. Because of its high prevalence, FGPs have been thought to be an incidental finding with little clinical significance in most patients. However, FGPs have important clinical significance in that they basically occur in gastric mucosa without atrophic gastritis or *H. pylori* infection[10,11]. This is quite different from other gastric polyps such as hyperplastic polyps or adenomas which have a strong link to *H. pylori* infection. When we find multiple FGPs in the stomach, we must be careful to rule out FAP, since FGPs arise in both sporadic and

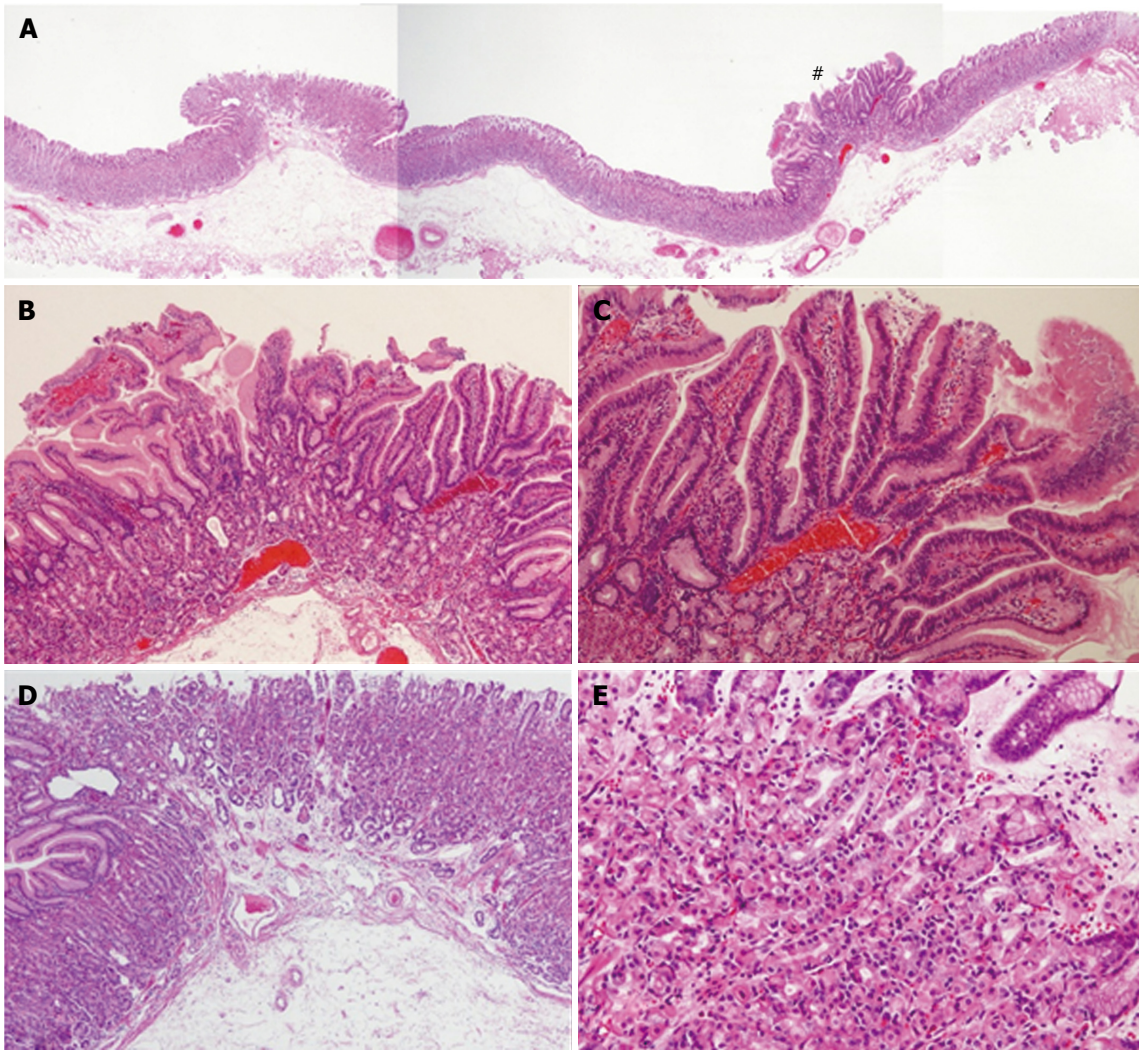

Figure 4 Histological examination of the endoscopic submucosal dissection specimen from Case 2. Shows that the right side lesion (#) is consistent with reddish polypoid and the left side lesion is consistent with isochromatic small polyp (A). High magnification of the right side lesion (#) shows irregularly branching tumor glands with atypical nuclei at the surface part of the lesion, and proliferation of fundic glands with some cystic dilatation at the basal part of the lesion, which was diagnosed as very well-differentiated adenocarcinoma occurring in fundic gland polyps (FGPs) (B and C). High magnification of the left side lesion shows proliferation of fundic glands with some cystic dilatation, which was diagnosed as FGP without dysplasia (D and E). FGP: Gastric fundic gland polyp.

systemic clinical settings associated with FAP patients. Moreover, systemic FGPs are more likely to be multiple polyps compared to the sporadic setting[12]. Although multiple FGPs were observed in the present two cases, we ruled out FAP by negative colonoscopy findings and no family history of FAP in either case.

Systemic FGPs often contain low-grade dysplasia in superficial foveolar epithelium with an incidence rate of 25%[2], but high-grade dysplasia and rarely invasive gastric adenocarcinoma have been reported[3,13]. In contrast, sporadic FGPs have been thought to be benign lesions with less malignant potential. Low-grade dysplasia was diagnosed in only 1% of sporadic FGPs (3 of 270 cases)[2]. There are only two reports showing that sporadic FGPs had high-grade dysplasia or adenocarcinoma[4,5].

Because it is extremely rare, there are few endoscopic findings of sporadic FGPs with dysplasia, and no reports of findings using ME-NBI.

Regarding the conventional endoscopic finding of tumor color in the present cases, it was only the FGPs with carcinoma that showed a reddish color, while several FGPs located at another site all showed isochromatic color. Maki *et al*[14] reported that reddish coloration by conventional endoscopy was a useful objective marker for differentiating cancerous lesions from superficial elevated lesions of the stomach. The appearance of reddish coloration in conventional endoscopy may therefore be useful for detecting and differentiating cancerous lesions from non-dysplastic FGPs. Furthermore, the ME-NBI findings of FGPs with cancer were completely different from those of FGPs without dysplasia. FGPs with cancer showed an irregular MV pattern plus irregular MS pattern with demarcation (vessels within epithelium pattern), whereas FGPs without dysplasia showed a regular

MV pattern and regular MS pattern with demarcation (epithelium within vessels pattern). These ME-NBI findings may accurately reflect the surface structure and tumor vessels of the adenocarcinoma component of foveolar type pathologically.

Considering the fate of this well-differentiated adenocarcinoma from sporadic FGP, there is no report of advanced adenocarcinoma from sporadic FGP. Therefore, we speculate that such cancer has less malignant potential, and may not increase in size or even diminish spontaneously. Furthermore, a recent report from systematic review and meta-analysis revealed that long-term use of PPIs therapy increase the risk of FGPs[15]. Although it is unclear whether dysplasia occurs in such PPIs associated FGPs[15], PPIs have been widely used in clinical practice. It is expected that we encounter FGPs more frequently in future, and may encounter FGPs with cancer in such cases.

In summary, we reported two cases of adenocarcinoma occurring in sporadic FGP. Reddish coloration by conventional endoscopy and ME-NBI findings were very useful for differentiating FGP with cancer from non-dysplastic FGP.

COMMENTS

Case characteristics

A 68-year-old man and a 63-year-old woman without apparent symptoms were referred to our institution. They had no medical history of proton pump inhibitor use and no family history.

Clinical diagnosis

Gastric fundic gland polyps (FGPs).

Differentiated diagnosis

Gastric hyperplastic polyps.

Laboratory diagnosis

Helicobacter pylori was negative by antibody in blood serum.

Imaging diagnosis

Suspicious for gastric adenocarcinoma of differentiated type by magnifying endoscopy with narrow band imaging (ME-NBI).

Pathological diagnosis

The final pathological diagnosis was very well-differentiated adenocarcinoma occurring in FGP.

Treatment

Resected by endoscopic submucosal dissection in both cases.

Related reports

There are only two reports showing that sporadic FGPs had high-grade dysplasia or adenocarcinoma.

Term explanation

FGPs are initially recognized as one of the gastric lesions in patients with familial adenomatous polyposis (FAP). In contrast, sporadic FGPs which are not associated with FAP, we frequently encounter in daily clinical practice.

Experiences and lessons

Reddish coloration by conventional endoscopy and ME-NBI findings were very useful for differentiating FGP with cancer from non-dysplastic FGP.

Peer-review

This is a well written manuscript describing the significance of endoscopic findings of sporadic FGPs with malignant potential.

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P- Reviewer: Kishikawa H, Viola LA **S- Editor**: Qi Y **L- Editor**: A
E- Editor: Zhang FF

World Journal of Gastroenterology

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Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.3748/wjg.v22.i40.9035

World J Gastroenterol 2016 October 28; 22(40): 9035-9038
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

Establishment of various biliary tract carcinoma cell lines and xenograft models for appropriate preclinical studies

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Author contributions: Ojima H wrote and revised this letter; Ojima H, Yamagishi S and Shibata T conducted the study and performed the data analyses; Shimada K obtained surgical biliary tract carcinoma specimens and performed the clinical data analyses; all authors read and approved the final manuscript.

Conflict-of-interest statement: Ojima H reports grants from Merck Serono Co., Ltd., grants from Eli Lilly Japan Co., Ltd., outside the submitted work.

Manuscript source: Invited manuscript

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Received: July 13, 2016
Peer-review started: July 16, 2016
First decision: August 19, 2016
Revised: September 8, 2016
Accepted: September 28, 2016
Article in press: September 28, 2016
Published online: October 28, 2016

Abstract

We recently reported several driver genes of biliary tract carcinoma (BTC) that are known to play important roles in oncogenesis and disease progression. Although the need for developing novel therapeutic strategies is increasing, there are very few BTC cell lines and xenograft models currently available for conducting preclinical studies. Using a total of 88 surgical BTC specimens and 536 immunodeficient mice, 28 xenograft models and 13 new BTC cell lines, including subtypes, were established. Some of our cell lines were found to be resistant to gemcitabine, which is currently the first choice of treatment, thereby allowing highly practical preclinical studies to be conducted. Using the aforementioned cell lines and xenograft models and a clinical pathological database of patients undergoing BTC resection, we can establish a preclinical study system and appropriate parameters for drug efficacy studies to explore new biomarkers for practical applications in the future studies.

Key words: Biliary tract carcinoma; Cell line; Xenograft model; Preclinical study

Core tip: Although the need for developing novel

therapeutic strategies for biliary tract carcinoma (BTC) is increasing, there are only few xenograft models and cell lines available for *in vivo* and *in vitro* studies, respectively. To conduct appropriate preclinical studies, we established 28 xenograft models and 13 new BTC cell lines using several surgical BTC specimens and immunodeficient mice. Using the aforementioned cell lines and xenograft models and a clinical pathological database of patients undergoing BTC resection, we can establish appropriate parameters for drug efficacy studies to explore new biomarkers for practical applications in the future studies.

Ojima H, Yamagishi S, Shimada K, Shibata T. Establishment of various biliary tract carcinoma cell lines and xenograft models for appropriate preclinical studies. *World J Gastroenterol* 2016; 22(40): 9035-9038 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i40/9035.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i40.9035

TO THE EDITOR

Biliary tract carcinoma (BTC) is an extremely malignant tumor. The incidence and mortality rates of BTC are currently rising and are particularly high in Asian countries. Surgical resection is the only curative treatment; however, most cases are diagnosed to be at advanced and inoperable stages by the time patients visit a hospital. The most serious problem is that there are no efficient chemotherapeutic regimens for patients with inoperable or recurrent BTC. Worldwide, gemcitabine-cisplatin combination therapy is the first choice, but clinicians are not satisfied with its efficacy. New drugs are needed for BTC patients.

Recently, we conducted genomic analyses of clinical specimens from 260 patients, which is the largest study till date, wherein we identified genomic abnormalities, which could be potential therapeutic targets, in 32 driver genes that play important roles in oncogenesis and disease progression in approximately 40% of BTC patients[1]. Although the need for developing novel therapeutic strategies is increasing, there are very few BTC-related resources currently available for conducting preclinical studies. The main reasons are as follows: the number of surgical BTC patients is not high at a single institute, and there is no large clinicopathological database. It is difficult to obtain surgical specimens for basic research. Therefore, there are only few xenograft models and cell lines available for *in vivo* and *in vitro* studies.

To conduct appropriate preclinical studies, surgical BTC specimens (collected from Japanese patients at the National Cancer Center Hospital, Tokyo, Japan since 2005 in an appropriate manner without any interference to pathological diagnosis) were directly transplanted into immunodeficient mice and subjected to cell culture medium to establish xenograft models and cell lines, respectively, as reported in 2010[2]. From a total of 88 BTC specimens and 536 immunodeficient mice during the period 2005-2013, we established 28 xenograft models (18 intrahepatic cholangiocarcinoma, four perihilar, and six distal BTC) and 13 new BTC cell lines, including subtypes (eight intrahepatic cholangiocarcinoma, two perihilar, and three distal BTC) (Table 1). Some of our established cell lines were found to be resistant to gemcitabine (Table 2), thereby allowing highly practical preclinical studies to be conducted. In addition, we conducted molecular pathology analyses of cell lines and constructed a clinical pathological database of patients undergoing BTC resection to establish appropriate parameters for drug efficacy studies to explore new biomarkers for practical applications (Figure 1)[2-5]. All experiments were approved by the Animal Care and Ethics Committee of the National Cancer Center (ID: T05-046). This study was approved by the Ethical Committee of the National Cancer Center (ID: 2007-022).

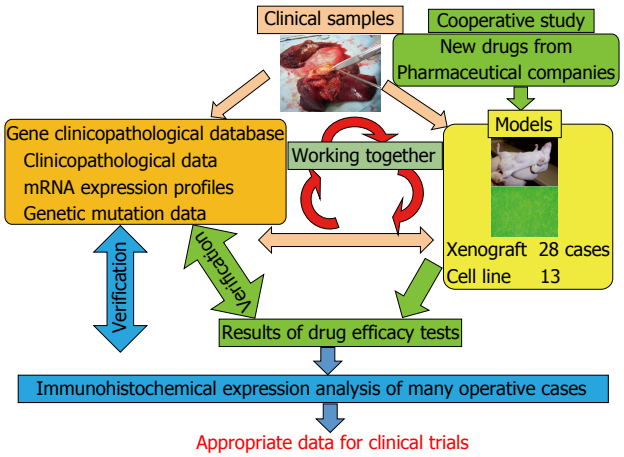

Figure 1 Relationship between our materials and databases. There are three key factors: clinical samples, databases, and biliary tract carcinoma (BTC) models. Both the models and the databases are derived from the clinical samples. These databases comprise "clinicopathological data", "mRNA expression profiles", and "genetic mutation data". BTC models are "xenograft models" and "cell lines". These models are used for cooperative studies with pharmaceutical companies for translational research. For example, they provide us with new anti-cancer drugs, and we can perform drug efficacy tests. If necessary, we can also perform an immunohistochemical expression analysis. Then, we can compare the results of the analysis with those in the databases and validate them. After these steps, we can provide appropriate data to clinicians. Together, these databases and materials make translational research far more detailed and suitable for clinical trials.

Preclinical studies have found very little evidence regarding the combined effects of prospective anticancer combination therapies, including gemcitabine. Therefore, we continue to examine the combined effects of the utility of the Bliss method and combination index to assess the prognosis of BTC. Moreover, we are going to release some of our resources and data in the near future. We believe that our materials and data will not only aid in conducting appropriate preclinical studies but

Table 1 Clinicopathological features of original biliary tract tumors

Xenograft	Pathological diagnosis of original tumor	Age/sex	Histologic type	Prognosis (survival days)	Chemotherapy	Clinical evaluation of chemotherapy effect (effective days)	Established cell line
1	CCC	70/F	Adeno, mod	Death (402)	Non		NCC-CC1
2	CCC	71/F	Adeno, mod	Death (175)	Non		NCC-CC3-1 NCC-CC3-2
3	CCC	59/M	Adeno, mod	Alive (2172)	Non		NCC-CC4-1 NCC-CC4-2 NCC-CC4-3(NCC-CC5)
4	CCC	31/M	Adeno, mod + PSC	Death (386)	GEM + TS1	SD (84 d)	NCC-CC6-1 NCC-CC6-2
5	Distal BDCa	58/F	Adeno, mod	Death (299)	GEM	PD	NCC-BD1
6	Distal BDCa	77/F	Adeno, mod	Death (393)	GEM	PD	NCC-BD2[1]
7	Distal BDCa	80/M	Adeno, mod	Death (212)	Non		NCC-BD3
8	Hilar BDCa	74/M	Adeno, mod	Death (172)	Non		NCC-BD4-1 NCC-BD4-2
9	Hilar BDCa	48/M	Adeno, well	Alive (500)	GEM	PD	NA
10	Hilar BDCa	43/M	Adeno, mod	Alive (1422)	Non		NA
11	CCC	69/M	Adeno, mod	Death (174)	Non		NA
12	CCC	54/F	Adeno, mod	Death (181)	Non		NA
13	CCC	56/M	Adeno, mod	Death (319)	GEM	PD	NA
14	CCC	73/M	Adeno, mod	Death (53)	Non		NA
15	CCC	54/M	Adeno, mod	Alive (2608)	Non		NA
16	CCC	45/F	Adeno, mod	Alive (882)	GEM + CDDP	Unknown	NA
17	CCC	72/M	Muc	Death (749)	GEM/GEM + TS1	Unknown	NA
18	CCC	78/M	Adeno, mod	Death (382)	GEM	Unknown	NA
19	CCC	66/M	Adeno, mod	Death (168)	Non		NA
20	CCC	65/M	CoCC	Alive (1604)	Non		NA
21	CCC	70/M	Adeno, por	Death (851)	GEM	SD (49 d)	NA
22	CCC	63/F	Adeno, mod	Alive (363)	Unknown	Unknown	NA
23	CCC	72/M	Adeno, mod	Death (394)	GEM	PD	NA
24	CCC	77/F	Adeno, mod	Death (445)	GEM	SD (105 d)	NA
25	Hilar BDCa	66/M	Adeno, mod	Alive (102)	GEM + TS1	Unknown	NA
26	Distal BDCa	54/M	Adeno, mod	Alive (2096)	Non		NA
27	Distal BDCa	67/M	Adeno, mod	Death (672)	GEM + TS1	PD	NA
28	Distal BDCa	80/M	Adeno, mod	Alive (2024)	GEM	PR-CR (548 d)	NA

[1]BD2 was obtained from the direct culture of patient specimens. CCC: Cholangiocellular carcinoma; BDCa: Bile duct carcinoma; Adeno: Adenocarcinoma; mod: Moderately differentiated; PSC: Primary sclerosing cholangitis; Muc: Mucinous carcinoma; CoCC: Cholangiolocellular carcinoma; por: Poorly differentiated; non: No chemotherapy received; GEM: Gemcitabine; CDDP: Cisplatin; SD: Stable disease; PD: Progressive disease; PR: Partial response; CR: Complete response.

Table 2 Sensitivity to gemcitabine in each cell line

Cell line	Sensitivity to gemcitabine in cell line[1]			
	IC_{50} (μmol/L)	IC_{60} (μmol/L)	IC_{70} (μmol/L)	IC_{80} (μmol/L)
NCC-CC1	86.78	N.A	N.A	N.A
NCC-CC3-1	0.04	1.82	9.31	85.21
NCC-CC3-2	0.10	1.92	43.83	N.A
NCC-CC4-1	0.05	4.08	N.A	N.A
NCC-CC4-2	0.03	11.53	N.A	N.A
NCC-CC4-3 (NCC-CC5)	0.06	4.92	95.10	N.A
NCC-CC6-1	0.01	0.02	0.06	3.76
NCC-CC6-2	10.98	35.67	N.A	N.A
NCC-BD1	7.66	58.00	N.A	N.A
NCC-BD2	N.A	N.A	N.A	N.A
NCC-BD3	N.A	N.A	N.A	N.A
NCC-BD4-1	0.04	0.06	0.09	2.93
NCC-BD4-2	0.06	0.07	0.19	5.37

[1]The cytotoxicity of gemcitabine for each cell line was assessed by a modified 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt assay with CellTiter 96 Aqueous One Solution Reagent (Promega, Madison, WI, United States). Tumor cells (3000 cells/well) in the exponential growth phase were grown in 96-well plates. IC: Inhibitory concentration.

also accelerate basic research of BTC.

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P- Reviewer: Cho YB, Peraldo-Neia C **S- Editor**: Qi Y
L- Editor: A **E- Editor**: Zhang FF

Published by **Baishideng Publishing Group Inc**
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ISSN 1007-9327