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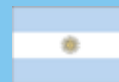
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^[1]Passed away on October 20, 2007

^[2]Passed away on June 11, 2007



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Surgical outcome of adenosquamous carcinoma of the pancreas

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Abstract

Adenosquamous carcinoma is rare, accounting for 3%-4% of all pancreatic carcinoma cases. These tumors are characterized by the presence of variable proportions of mucin-producing glandular elements and squamous components, the latter of which should account for at least 30% of the tumor tissue. Recently, several reports have described cases of adenosquamous carcinoma of the pancreas. However, as the number of patients who undergo resection at a single institute is limited, large studies describing the clinicopathological features, therapeutic management, and surgical outcome for adenosquamous carcinoma of the pancreas are lacking. We performed a literature review of English articles retrieved from Medline using the keywords 'pancreas' and 'adenosquamous carcinoma'. Additional articles were obtained from references within the papers identified by the Medline search. Our subsequent review of the literature revealed that optimal adjuvant chemotherapy and/or radiotherapy regimens for adenosquamous carcinoma of the pancreas have not been established, and that curative surgical resection offers the only chance for long-term survival. Unfortunately, the prognosis of the 39 patients who underwent pancreatic resection for adenosquamous carcinoma was very poor, with a 3-year overall survival rate of 14.0% and a median survival time of 6.8 mo. Since the postoperative prognosis of adenosquamous carcinoma of the pancreas is currently worse than that of pancreatic adenocarcinoma, new adjuvant chemotherapies and/or radiation techniques should be investigated as they may prove indispensable to the improvement of surgical outcomes.

INTRODUCTION

The majority of malignant tumors in the pancreas are adenocarcinomas. Adenosquamous carcinoma of the pancreas occurs less frequently with an incidence of 3%-4%^[1]. These tumors are a malignant epithelial carcinoma of the pancreas and are characterized by the presence of variable proportions of both glandular and squamous components. At least 30% of the neoplasm should be comprised of the squamous component^[1,2]. Recently, several reports have described cases of adenosquamous carcinoma of the pancreas^[3-6]. However, as the number of patients who undergo resection at a single institute is limited, large studies describing the clinicopathological features, therapeutic management and surgical outcome for adenosquamous carcinoma of the pancreas are lacking.

To the best of our knowledge, our survey of the English literature reporting on adenosquamous carcinoma of the pancreas, which was found on Medline, revealed that only 39 intent-to-cure surgical resections had been performed and had clearly presented data (Table 1)^[7-28]. The purpose of this study was to clarify the surgical outcome including survival rates after surgery, and to determine the prognostic factors of adenosquamous carcinoma of the pancreas by conducting a retrospective analysis of the 39 patients.

PATIENTS

Our survey of the literature from 1980 to the end of 2007 revealed that 45 patients underwent surgical resection for adenosquamous carcinoma of the pancreas^[7-28].

Table 1 Clinical and pathological data for the 39 cases that underwent surgical resection for adenosquamous carcinoma of the pancreas

Author	Ref	Yr	Age	Sex	Location	Surgery	Size (cm)	Cx	RT	Rec site	Survival
Ishikawa	7	1980	67	M	Body	DP	10.0	-	-	Widespread metastasis	4 mo
			53	F	Head	PD	4.2	-	-		2 d
			61	M	Head	PD	4.5	-	-		12 mo
Wilczynski	8	1984	68	M	Head, body	PD	4.5	-	-		20 d
Yamaguchi	9	1991	60	M	Head	PD	ND	-	-	ND	3 mo
			52	F	Head	PD	ND	-	-	ND	7 mo
			44	F	Head	PD	ND	-	-	ND	5 mo
			56	F	Head	PD	ND	-	-	ND	4 mo
			56	M	Head	PD	ND	-	-	ND	5 mo ¹
			68	F	Head	PD	ND	-	-	ND	5 mo ¹
			49	F	Body	DP	ND	-	-	ND	5 mo
			61	M	Tail	DP	ND	-	-	ND	14 mo ¹
Motojima	10	1992	52	M	Body, tail	DP	7.0	ND	ND	Systemic metastasis	3 mo
			75	M	Head	PD	3.0	ND	ND	Liver	10 mo
			75	F	Head	PD	6.0	ND	ND	Liver	8 mo
Tanaka	11	1994	48	F	Head	PD	4.2	+	-	ND	7 mo
Makiyama	12	1995	58	M	Head	PD	5.0	-	-	Peritoneum	18 mo
Onoda	13	1995	64	M	Body, tail	DP	7.0	+	-	Liver, peritoneum	3 mo
Campman	14	1997	65	F	Body, tail	DP	7.5	ND	ND	ND	ND
Kuji	15	1997	73	M	Body, tail	TP	6.0	-	-	ND	2 mo
Nabae	16	1998	60	M	Body	DP	6.0	-	IOR	ND	4 mo
			73	M	Head	PD	ND	-	-	Liver	10 mo
Myung	17	1998	64	M	Head	PD	3.5	-	-	ND	4 mo ¹
Lozano	18	1998	75	M	Head, body	PD	4.5	+	+	ND	ND ¹
			42	M	Head	PD	3.5	+	+	ND	ND ¹
Aranha	19	1999	52	M	Head	PD	3.2	+	+	Systemic metastasis	13 mo
			62	M	Head	PD	3.0	+	+	Systemic metastasis	14 mo
Komatsuda	20	2000	67	M	Body	DP	5.0	-	-	Peritoneum	6 mo
Yavus	21	2000	51	M	Head	PD	4.0	ND	ND	-	36 mo ¹
			48	M	Head	PD	2.0	ND	ND	ND	ND
Yamaue	22	2001	63	F	Head	PD	4.5	+	+	-	40 mo ¹
Kardon	23	2001	ND	ND	Head	PD	ND	-	-	ND	33 mo ¹
Murakami	24	2003	41	M	Head	PD	3.0	-	+	Peritoneum	5 mo
Rahemtullah	25	2003	ND	ND	Head	PD	ND	ND	ND	ND	13 mo ¹
			ND	ND	Head	PD	ND	ND	ND	ND	ND
Alwaheeb	26	2005	45	M	Head	PD	6.0	-	-	ND	ND
Hsu	27	2005	66	M	Head	PD	3.5	-	-	ND	2.5 mo
			38	F	Head	PD	3.8	-	+	ND	6.8 mo
Jamali	28	2007	75	M	Head	PD	3.0	+	-	Liver	6 mo

Cx: Chemotherapy; RT: Radiotherapy; Rec site: Recurrence site; ND: Not described; PD: Pancreaticoduodenectomy; DP: Distal pancreatectomy; TP: Total pancreatectomy. ¹Surviving patients.

Of these, six patients were excluded due to a lack of clear data. The remaining 39 patients were analyzed in this study (Table 1) and included 25 men, 11 women, and three patients of unknown sex with a mean age of 59.0 years (range, 38-75 years). The prognosis outcome of each case was obtained from the published data. The clinicopathological data associated with the pancreatic adenosquamous carcinomas described in these case reports were evaluated, and included tumor location, type of operation, tumor size, whether chemotherapy and radiotherapy had been administered, recurrence sites, and survival times. All of the patients had undergone surgery involving an attempted curative resection. Survival rates were generated using the Kaplan-Meier method and compared using the log-rank test^[29]. Values were expressed appropriately as the mean \pm SD. Differences in proportions were evaluated by the Pearson chi-square test. A value of $P < 0.05$ was considered to be statistically significant.

DIAGNOSIS OF ADENOSQUAMOUS CARCINOMA OF THE PANCREAS

Table 1 lists 39 patients who had undergone surgical resection for adenosquamous carcinoma of the pancreas. Adenosquamous carcinomas have not been associated with any specific clinical syndromes^[2,30]. Each of the 39 patients presented clinical symptoms such as abdominal pain, back pain, painless jaundice, anorexia, and/or body weight loss (data not shown). Accurate preoperative diagnosis of adenosquamous carcinoma of the pancreas is very difficult, because imaging studies have revealed no characteristic features that can facilitate the differentiation of this tumor type from ordinary invasive ductal carcinoma. One study reported that intense Gallium-67 citrate uptake was observed in adenosquamous carcinoma of the pancreas, indicating that Gallium-67 citrate scintigraphy might be useful in detecting these carcinomas^[15]. However, more detailed

imaging data are required to improve the ability to diagnose this rare disease.

Adenosquamous carcinoma of the pancreas appears to be larger than ordinary pancreatic adenocarcinoma. The tumors in the 27 cases for which the relevant data was available had a mean size of 4.8 ± 1.8 cm (range, 2-10 cm; Table 1). Preoperative cytological or pathological diagnosis of adenosquamous carcinoma of the pancreas is reportedly rare^[12,16-18,21,24,26,30]. However, the two malignant cellular components of adenosquamous carcinoma can be recognized in aspirated smears^[17,18,24]. A careful search for glandular differentiation is warranted when the squamous component predominates, particularly if squamous carcinoma specimens only are obtained by biopsy or fine needle aspiration biopsy^[12,16]. Adenosquamous carcinoma of the pancreas has no specific radiological findings or serum data, including tumor markers such as carcinoembryonic antigen, carbohydrate antigen 19-9, or squamous cell carcinoma antigen^[12,22]. Physicians should try to remember to consider adenosquamous carcinoma of the pancreas in the differential diagnosis of ordinary pancreatic adenocarcinoma, especially if the patient has severe abdominal symptoms and/or a large tumor size^[2,30]. Recently, preoperative and intraoperative cytological examinations have been diagnostically correct, however these findings did not alter treatment decisions or survival^[30].

MANAGEMENT FOR RESECTABLE ADENOSQUAMOUS CARCINOMA OF THE PANCREAS

Since adenosquamous carcinomas are uncommon tumors with a poor prognosis, the outcomes associated with various therapeutic interventions are not well defined.

Table 1 lists the tumor location and operative method used in the 39 cases analyzed here. Three main operative methods were performed: pancreaticoduodenectomy (PD) including pylorus-preserving PD (PPPD) in 30 cases (76.9%); distal pancreatectomy (DP) in eight cases (20.5%); and total pancreatectomy (TP) in one case (2.6%). Tumors were located in the head alone in 28 cases (76.9%), in the head and body in two cases, and in the body and/or tail of the pancreas in nine cases (23.1%). Although adenosquamous carcinoma of the pancreas has different clinicopathological features to pancreatic adenocarcinoma, the treatment strategy of patients with adenosquamous carcinoma is dealt with in the same manner as patients with adenocarcinoma. Surgical treatment remains the only curative management option that is seriously considered for adenosquamous carcinoma of the pancreas.

To date, only eight patients have received adjuvant chemotherapy, indicating that postoperative adjuvant chemotherapy is not usually administered to patients with adenosquamous carcinoma of the pancreas (Table 1). Tanaka *et al* reported that the size of an unresectable adenosquamous carcinoma of the pancreas was reduced by neo-adjuvant chemotherapy consisting of a combination of interferon- α , tumor necrosis factor- α , and 5-fluoroura-

cil^[11]. However, the patient only survived 7 mo after surgery^[11]. In this case, although neo-adjuvant chemotherapy might not have contributed to prolonging the patient's survival, the ability of the chemotherapy to reduce the size of the tumor from one that was unresectable to one that could be resected was confirmed. In the current study, the adjuvant chemotherapy group had a 2- or 3-year cumulative survival rate of 16.7% and a median survival period of 7 mo (Table 2). In comparison, the group who did not receive adjuvant chemotherapy had a 2-year cumulative survival rate of 9.2% and a median survival period of 5 mo ($P = 0.364$). Almost all of the patients in the adjuvant chemotherapy group were treated with a 5-fluorouracil-based regimen. Recently, adjuvant chemotherapy using new drug agents has been considered as the standard therapeutic option following resection for pancreatic adenocarcinoma, and several reports suggest that adjuvant chemotherapy with gemcitabine is responsible for a significant increase in patient survival^[31-33]. Postoperative administration of gemcitabine also significantly delayed the development of recurrent disease after complete resection of pancreatic cancer compared with observation alone^[34]. However, information regarding gemcitabine use in cases with adenosquamous carcinoma of the pancreas is not available as previous reports lack such data. Further investigations examining whether adjuvant chemotherapy using gemcitabine will improve surgical outcome in patients with adenosquamous carcinoma of the pancreas are therefore warranted.

There are no published prospective randomized controlled trials investigating radiotherapy treatment of pancreatic adenosquamous carcinoma following curative resection, only retrospective studies. Limitations of the present study include the errors and biases inherent in a small retrospective study design. Two retrospective studies investigating the benefit of radiotherapy following curative resection for pancreatic carcinoma showed no significant difference in the overall survival between patients who were or were not treated with radiotherapy^[35,36]. In the current study, patients who had received intra- and/or postoperative radiotherapy had a 2- or 3-year cumulative survival rate of 20.0% and a median survival period of 13 mo (Table 2). By comparison, the non radiotherapy group had a 2-year cumulative survival rate of 9.0% and a median survival period of 6 mo ($P = 0.284$). There was no significant difference in survival between patients who did and did not receive radiotherapy.

PROGNOSIS AFTER PANCREATIC RESECTION

The overall 1-, 2-, and 3-year survival rates after pancreatic resection were 25.5%, 14.0%, and 14.0%, respectively (Figure 1). Table 1 shows operative mortality occurred in two patients during the early 1980s^[7,8]. One patient died of myocardial infarction 2 d after undergoing PD and another died of numerous postoperative complications including electrolyte disturbance from massive abdominal fluid

Table 2 Clinical characteristics after surgical resection for adenosquamous carcinoma of the pancreas

Characteristics	No. of patients	Survival rate (%)			Median survival in months (range)	P value
		1 yr	2 yr	3 yr		
Overall	39	25.5	14.0	14.0	6.8 (4.6-9.0)	
Age (yr)						
< 60	16	26.9	9.0	9.0	6.8 (4.4-9.2)	0.975
> 60	20	20.4	13.6	13.6	6.0 (1.3-10.7)	
Gender						
Male	25	28.4	8.5	8.5	6.0 (1.1-10.9)	0.842
Female	11	12.0	12.0	12.0	6.8 (5.0-8.6)	
Tumor location						
Head	30	34.8	17.9	17.9	8.0 (5.3-10.7)	0.017
Body or tail	9	11.1	-	-	4.0 (2.6-5.4)	
Operation type						
PD	30	33.4	17.2	17.2	8.0 (5.2-10.8)	0.063
DP or TP	9	12.5	-	-	4.0 (2.7-5.3)	
LN metastasis						
Present	14	20.0	-	-	5.0 (2.0-8.0)	0.134
Absent	8	50.0	50.0	50.0	5	
Chemotherapy						
Yes	8	50.0	16.7	16.7	7.0 (0.0-15.4)	0.364
No	23	18.4	9.2	-	5.0 (3.0-7.0)	
Radiotherapy						
Yes	7	60.0	20.0	20.0	13.0 (0.0-26.3)	0.284
No	23	18.0	9.0	-	6.0 (4.0-8.0)	

PD: Pancreaticoduodenectomy; DP: Distal pancreatectomy; TP: Total pancreatectomy; LN metastasis: Lymph node metastasis.

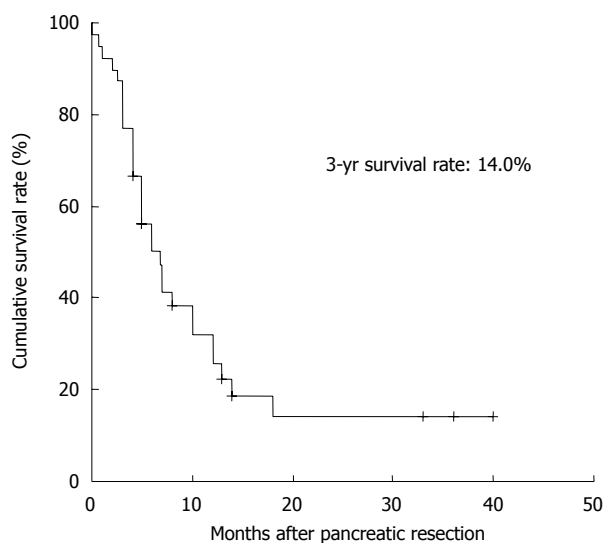


Figure 1 Survival after surgical resection for adenosquamous carcinoma of the pancreas ($n = 39$).

losses, acute renal failure and eventually congestive heart failure 20 d after undergoing PD^[7,8]. Univariate analysis of the different prognostic factors predicted to contribute to patient prognosis showed that tumor location was the only unfavorable prognostic factor. Median survival of patients with a tumor located in the body and/or tail (4 mo) was significantly worse than those with tumors located in the head (8 mo) (Table 2). Prognostic differences based on tumor location may relate to tumor size, as the size of a distal pancreatic tumor (7.3 ± 1.8 cm) was significantly larger than that of a proximal pancreatic tumor (4.7 ± 1.9 cm, $P = 0.002$). Age, gender, type of operative procedure, and

lymph node metastasis were not significant prognostic factors.

Recently, long-term survival after PD for pancreatic adenocarcinoma has improved, and the number of patients surviving for five or 10 years has increased^[37-39]. On the other hand, the prognosis for the 39 patients with adenosquamous carcinoma in this study was poor, with a 3-year overall survival rate of only 14.0%, and includes two patients with hospital mortality. A patient surviving for five years post-resection has not been reported yet (Table 1). This suggests that adenosquamous carcinoma of the pancreas has greater malignant potential than adenocarcinoma of the pancreas. A previous report also found that squamous cell carcinomas grow at twice the speed of adenocarcinomas^[31]. Therefore, once an adenocarcinoma has transformed into an adenosquamous carcinoma, the carcinoma may exhibit a higher degree of malignancy^[40].

CONCLUSION

Even though curative resection for adenosquamous carcinoma of the pancreas was performed in the 39 patients, prognosis remained poor because systemic metastases in the liver and peritoneal dissemination were the major sites of recurrence (Table 1). In addition, tumor recurrence occurred during the early stages of the post-operative period in a large number of patients. Yamaue et al. reported that it might be preferable not to perform a pancreatic resection if a pancreatic tumor is diagnosed as an adenosquamous carcinoma^[22]. Consensus of opinion regarding the surgical indication required for this type of tumor has not been reached yet. Elucidating

a surgical treatment strategy based on the appropriate surgical indication is essential for improving the surgical outcome of adenosquamous carcinoma of the pancreas.

The results of this current study indicate that tumor location may be an important factor in determining the appropriate surgical indication. Namely, surgical resection may be better suited for proximal pancreatic tumors than for distal tumors because the proximal location of tumors was the only significant favorable prognostic factor found in this study. Furthermore, exploration of new radiation techniques and chemotherapeutic regimens using new drug agents such as gemcitabine may be required because conventional chemotherapy and radiotherapy treatments did not contribute to survival benefit. The incorporation of novel 'molecularly targeted' agents into therapy will also be required to improve surgical outcome.

Although adenosquamous carcinoma of the pancreas has a poor prognosis even after curative resection, we must continue to endeavor to improve the surgical outcome of this tumor, because despite its rarity, it occurs worldwide. More data, including epidemiological and pathological findings, will be required to determine the appropriate surgical indication for this tumor.

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Adult eosinophilic gastroenteritis and hypereosinophilic syndromes

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Abstract

Eosinophilic gastroenteritis (EGE) in the adult is a distinctive pathologically-based disorder characterized by an eosinophil-predominant mucosal inflammatory process. Most often, the disorder is detected during endoscopic investigation for abdominal pain or diarrhea. Other causes of gastric and intestinal mucosal eosinophilia require exclusion, including parasitic infections and drug-induced causes. Occasionally, the muscle wall or serosal surface may be involved. EGE appears to be more readily recognized, in large part, due to an evolution in the imaging methods used to evaluate abdominal pain and diarrhea, in particular, endoscopic imaging and mucosal biopsies. Definition of EGE, however, may be difficult, as the normal ranges of eosinophil numbers in normal and abnormal gastric and intestinal mucosa are not well standardized. Also, the eosinophilic inflammatory process may be either patchy or diffuse and the detection of the eosinophilic infiltrates may vary depending on the method of biopsy fixation. Treatment has traditionally focused on resolution of symptoms, and, in some instances, eosinophil quantification in pre-treatment and post-treatment biopsies. Future evaluation and treatment of EGE may depend on precise serological biomarkers to aid in definition of the long-term natural history of the disorder and its response to pharmacological or biological forms of therapy.

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Key words: Eosinophils; Eosinophilic gastroenteritis; Eosinophilic gastritis; Eosinophilic enteritis

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INTRODUCTION

Eosinophilic gastroenteritis (EGE) is a distinct eosinophil-predominant inflammatory process in gastric or small intestinal mucosal biopsies. Although most published material has focused largely on the pediatric population, this manuscript focuses on the adult with EGE. Most often in adults, EGE is detected during endoscopic investigation for abdominal pain or diarrhea^[1-4]. Although the endoscopic changes are non-specific (Figure 1), chronic or recurring symptoms are present and other causes of intestinal eosinophilia require exclusion (e.g. parasitic infections, medications)^[1-4]. The mucosa of the stomach, intestine, or both may be involved most frequently. In one specific classification schema that included different forms of intestinal eosinophilic infiltration, involvement of the muscular layer or serosa was also described, but even in these, concomitant mucosal involvement was often present^[5].

EGE has been considered an uncommon, even rare disorder but this may well depend on its definition as well as the method of detection. In a single clinical practice, only less than 1% of all upper endoscopic studies during an 8-year period showed changes that led to a diagnosis of EGE^[6]. Biopsies are now commonly done during routine endoscopic evaluation, even if the mucosa is visually normal or if only non-specific changes are present. Finally, it is speculated that there may be some environmental factors (or allergen) in the diet or air that plays a critical role in the emergence of an eosinophil-predominant mucosal inflammatory process.

HISTOPATHOLOGICAL CRITERIA

Most often, the diagnosis of EGE is defined by histological evaluation of endoscopic biopsies. There are some potential diagnostic issues and pitfalls. First,



GE clinic

Figure 1 Endoscopic views of gastric antrum and gastric body showing non-specific erythematous and thickened mucosal folds with pre-pyloric pseudopolypoid change.

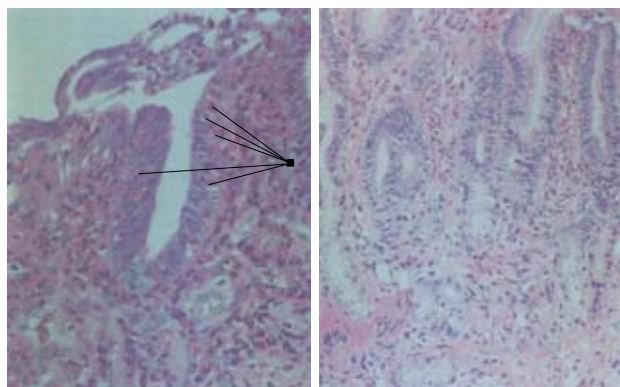


Figure 2 Photomicrographs show a formalin-fixed biopsy on the left with arrows delineating intraepithelial eosinophils along with numerous eosinophils in the lamina propria, while a Bouin's fixed biopsy on the right from an adjacent endoscopic biopsy shows a paucity of eosinophils..

pathologists, like endoscopists, have varying levels of expertise in mucosal biopsy interpretation. It is appreciated that there may be many “shades of grey” in the definition of EGE and the experience of the pathologist may determine disease recognition. To date, however, precise information on intra- and inter-observer error in the interpretation of biopsies in EGE is still needed. Second, eosinophils may normally be detected in the gastric and intestinal mucosa (as opposed to the normal esophagus), and only limited numbers of studies (mainly in children) have tried to quantify normal compared to abnormal numbers in health and different inflammatory disease states, e.g. ulcerative colitis^[7,8].

Third, these studies are also limited, to some degree, by the inherent “patchy” nature of the eosinophilic inflammatory process in EGE since the numbers of eosinophils may differ in biopsies obtained from different sites. Finally, fixation methods may be critical in the definition of eosinophils in gastric and intestinal biopsies. For example, Bouin's solution (often used for gastric or intestinal biopsies), can result in “bleaching” of eosinophil granules making detection much more difficult. If EGE is suspected, routine formalin fixation has been shown to provide more optimal material for routine staining (Figure 2)^[9].

CLINICAL FEATURES

Prior studies have suggested that EGE is a male-predominant clinical disorder^[1]. Some believe that clinical features may reflect extent, location and depth of infiltration of this eosinophilic inflammatory process within the gastrointestinal tract^[1,5]. Abdominal pain and diarrhea are common. Weight loss may occur, in part related to malabsorption. Iron deficiency associated with blood loss as well as protein-losing enteropathy may also be seen. If muscular layers are involved, obstruction or, even an acute abdomen has been recorded^[1-4], while serosal involvement may be associated with evidence of ascites^[5]. Peripheral blood eosinophilia has been recorded in up to 70%, but this is not specific for EGE and should lead to exclusion of other disorders, specifically parasitic infections^[3]. In some with EGE, increased serum IgE levels may be seen, but this is also not specific. Endoscopic evaluation might permit definition of the extent of the inflammatory process in the upper gastrointestinal tract. In rare reports^[10,11], celiac disease has been linked to EGE but as completely independent disorders.

Treatment is largely aimed at resolving symptoms. Medications used in EGE are largely based on empiric observation and experience. Because of the rarity of EGE, there are no controlled treatment trials available. Steroids have been used as a traditional form of therapy to reduce the inflammatory process^[1-4], however, these may cause steroid-related effects, especially because of their recurring need over prolonged periods. Other remedies have been used but their effectiveness still requires definition. These include: proton pump inhibitors^[5], mast cell stabilizers^[12,13], ketotifen^[9,14], leukotriene antagonists^[15], octreotide^[16] and surgical resection of involved intestinal segments^[17]. This lengthening therapeutic list might be construed as a clear reflection of the limited forms of effective therapy that are currently available.

HYPEREOSINOPHILIC SYNDROMES

The hypereosinophilic syndromes (HES) represent a heterogeneous group of rare disorders that have been defined in the past by persistent blood eosinophilia for more than 6 mo with evidence of organ involvement. The cause is unknown. HES may include a broad

spectrum of disorders, including familial or genetically-based eosinophilia and more sinister neoplastic disorders, including eosinophilic leukemia^[1,19]. About 25% of cases with HES, however, have eosinophilic infiltration in the gastrointestinal tract, and in some, this inflammatory process is reportedly localized only in gastric or intestinal mucosa.

The onset of HES is generally described between ages of 20 and 50 years with a male predominance^[1]. Abdominal pain and diarrhea with malabsorption have been described. In comparison with those with disease localized in some other non-intestinal sites, involvement of the intestinal tract has been associated with a limited prognosis, lympho-proliferative disorders^[20], and, in some, a fatal outcome^[1]. Unfortunately, there may be little to distinguish EGE and HES, especially if the latter is early in the clinical course and localized to the gastric and intestinal mucosa alone. In some, steroids, immune-suppressants and even biological agents have been used^[21]. Clearly, long-term clinical studies are needed to define and elucidate the natural history of EGE, a relatively unique inflammatory process, and to determine if there is a potential HES risk.

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REVIEW

Practical guidelines for diagnosis and early management of drug-induced liver injury

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Abstract

The spectrum of drug-induced liver injury (DILI) is both diverse and complex. The first step in diagnosis is a suspicion of DILI based on careful consideration of recent comprehensive reports on the disease. There are some situations in which the suspicion of DILI is particularly strong. Exclusion of other possible etiologies according to the pattern of liver injury is essential for the diagnosis. In patients with suspected DILI, diagnostic scales, such as the Councils for International Organizations of Medical Sciences/Roussel Uclaf Causality Assessment Method (CIOMS/RUCAM) scale, may be helpful for the final diagnosis. Early management of DILI involves prompt withdrawal of the drug suspected of being responsible, according to serum levels of alanine aminotransferase (ALT), alkaline phosphatase (ALP), and total bilirubin (T-Bil). However, as DILI patients may show resolution of liver injury without discontinuation of the drug, it should be carefully evaluated whether the suspected drug should be discontinued immediately with adequate consideration of the importance of the medication.

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INTRODUCTION

Drug-induced liver injury (DILI) is a common liver disease which generally occurs between 5 and 90 d after drug ingestion. The clinical picture of the disease is variable, ranging from transient mild elevation of liver enzymes to fulminant liver failure leading to death. DILI has been reported to be a cause of fulminant liver failure in 13%-30% of cases^[1-3]. DILI is divided into three types: hepatocellular, cholestatic, and mixed according to the Councils for International Organizations of Medical Sciences (CIOMS)^[4,5]. Hepatocellular type is defined by alanine aminotransferase (ALT) > 2 ULN (upper limits of normal) or $R \geq 5$, where R is the ratio of serum activity of ALT/serum activity of alkaline phosphatase (ALP), both of which are expressed as multiples of the ULN. Liver injury is likely to be more severe in hepatocellular type than in cholestatic/mixed type, and patients with elevated bilirubin levels in hepatocellular liver injury indicating serious liver injury with fatalities, are found at a rate of 0.7 to 1.3/100 000 individuals receiving a given drug^[2]. Cholestatic type is defined by $ALP > 2$ ULN or $R \leq 2$ and mixed type is defined by $ALT > 2$ ULN and $2 < R < 5$. Patients with cholestatic/mixed type are likely to develop chronic disease more frequently than those with hepatocellular type^[6]. For most drugs, the risk of liver injury is estimated to be 1-10/100 000 persons exposed. A recent report indicated that DILI occurs in 1/100 patients hospitalized in internal medicine departments^[7]. Thus, DILI is not a rare condition and sometimes leads to serious disease. Rapid and accurate diagnosis of DILI is important in daily practice. However, diagnosis of DILI is not easy and is mainly based on circumstantial evidence. As there is no gold standard for diagnosis, it is essential to exclude other possible etiologies for accurate diagnosis. A number of scoring systems have been proposed,

but even experts may make different judgments using these systems^[8]. This review summarizes recent trends regarding DILI and proposes practical guidelines for its diagnosis and early management.

RECENT REPORTS ON DILI

A recent report on DILI based on the database of the World Health Organization (WHO) indicated that the number of cases of DILI has been increasing since the 1990s^[9]. The WHO began monitoring adverse drug reactions in 1968, and there are more than 3 million reports on their database (<http://www.who-umc.org>). This large database is useful for obtaining information on previous reports regarding adverse reactions to drugs. Acetaminophen, drugs against human immunodeficiency virus (HIV), troglitazone, anti-convulsants (such as valproate), analgesics, antibiotics, and anti-cancer drugs are common causative agents of DILI with fatalities (Table 1)^[9]. Therefore, particular attention should be paid to patients taking one or more of these drugs who show liver injury. Analysis of 461 cases in Spain over a 10-year period indicated that amoxicillin/clavulanate was the most common drug involved in DILI (59/461 cases, 12.8%)^[10]. Moreover, in addition to amoxicillin/clavulanate, they reported that bentazepam, atorvastatin, and captopril were frequent causative drugs leading to chronic liver damage^[6]. In a retrospective study in Italy, hydroxymethylglutaryl-CoA reductase inhibitors were the most frequent causative drugs among 1069 cases of DILI (4.5% of cases of adverse drug reactions)^[11]. Other studies also showed acetaminophen, anti-retroviral therapy, antibiotics, lipid-lowering drugs, and anti-convulsants to be responsible for DILI^[12-18]. In recent analyses in Asia, traditional alternative medicines were reported to be the most common causes of DILI, in contrast to those in Western countries^[19]. Table 2 summarizes the drugs suspected to be responsible for DILI and the types of liver injury reported in the literature from various regions^[3,6,7,10,12,17-19]. In general, antibiotics, non-steroidal anti-inflammatory drugs, and anti-convulsants are frequent causative drugs of DILI. Importantly, although not shown in Table 2, two or more drugs were suspected to be responsible for DILI in about 10% of cases^[10,13]. Furthermore, it is notable that the incidences of DILI caused by herbal remedies or traditional medicines have been increasing over the last decade. The causative drugs for DILI are therefore becoming more diverse and complex. The first and most important step in managing cases of suspected DILI is to gain a detailed understanding of the causative drugs. In the United States of America, the Food and Drug Administration (FDA) records drug toxicity (<http://www.fda.gov/medwatch>), and the Drug Induced Liver Injury Network was established in 2003 to collect data on DILI in a prospective manner^[1]. A similar network is also in place in Spain^[6]. A worldwide network which collects all the reports on adverse drug reactions is needed to provide comprehensive information on DILI,

Table 1 Common causative agents of drug-induced liver injury with fatalities fatalities

Drug	n (%)
Acetaminophen	305 (16.9)
Anti-HIV ¹	
Stavudine, didanosine, nevirapine, zidovudine	303 (16.8)
Troglitazone	211 (11.7)
Anticonvulsants (valproate, phenytoin)	187 (10.3)
Anti-cancer	223 (12.3)
Flutamide	59 (3.3)
Cyclophosphamide	56 (3.1)
Methotrexate	55 (3.0)
Cytarabine	53 (2.9)
Antibiotics	158 (8.7)
Trovaflaxacin	57 (3.2)
Sulfa/trimethoprim	52 (2.9)
Clarithromycin	51 (2.8)
Anesthetic	
Halothane	85 (4.8)
Anti-tuberculosis	
Isoniazid	57 (3.2)
Diclofenac	56 (3.1)
Oxycodone	56 (3.1)

¹human immunodeficiency virus.

which would facilitate accurate diagnosis and early management.

PRACTICAL DIAGNOSIS OF DILI

Situations in which DILI should be suspected

In daily clinical practice, DILI can always be a cause of liver injury in patients taking medications. However, there are some situations in which DILI should be particularly suspected and are as follows^[20]: (1) Start of a new drug in the past 3 mo, (2) Presence of rash or eosinophilia, (3) Mixed type (hepatocellular and cholestatic) liver injury, (4) Cholestasis with normal hepatobiliary imaging and (5) Acute or chronic hepatitis without autoantibodies or hypergammaglobulinemia. Although DILI cannot be excluded if patients with any type of liver injury do not meet these criteria, their consideration may lead to early diagnosis of DILI.

Risk factors for DILI

Recognition of risk factors provides clues for the diagnosis of DILI, and some scoring systems include these elements. Host factors which may be associated with DILI are listed in Table 3. Age, gender, pregnancy, and alcohol intake are estimated as risk factors for patients, and liver injury with these risk factors is thought to be related to acute cytolytic hepatitis^[21]. In a recent analysis, age was reported to be the most important determinant in biochemical expression of amoxicillin/clavulanate hepatotoxicity, probably because of the slower drug elimination related to advanced age^[22]. In contrast, adverse events associated with valproate or erythromycin are more common in childhood^[23]. On the other hand, a retrospective study indicated that most patients with drug-induced acute liver failure undergoing

Table 2 Drugs suspected of being responsible for at least two cases of drug-induced liver injury and the types of liver injury reported in recent literature

Use	Drugs	Hepatocellular	Cholestatic	Mixed
Anti-microbial	Amoxicillin-clavulanate	28	26	23
	Azithromycin	0	8	0
	Trovaflaxacin	5	0	1
	Erythromycin	2	4	3
	Clindamycin	2	0	0
	Nitrofurantoin	1	1	0
	Levofloxacin	0	0	1
	Ciprofloxacin	2	1	1
	Flucloxacillin	0	7	1
	Sulfasalazine	1	0	1
	INH + RIP + PIZ	24	6	32
	HAART	4	1	1
	Dapsone	2	0	0
Anti-inflammatory	Acetaminophen	40	0	0
	Diclofenac	18	8	3
	Nimesulide	7	2	0
	Ibuprofen	8	3	9
Anti-convulsant	Carbamazepine	6	1	3
	Valproic acid	4	1	3
	Benzazepam	5	0	2
Psychiatric	Paroxetine	4	1	2
	Disulfiram	2	0	0
	Tetrabamate	6	1	0
Anti-cancer	Flutamide	12	1	5
	Methotrexate	3	0	0
Lipid-lowering	Atorvastatin	6	2	2
	Fenofibrate	1	0	2
Gastrointestinal	Ebrotidine	23	0	2
For circulation	Captopril	1	0	1
Anti-coagulant	Ticlopidine	8	5	1
For endocrine	Thiamazole	1	4	0
Immunosuppressant	Azathioprine	5	4	2
Others	Medical herbs	26	3	2
	OTC health supplements	3	0	0

INH: Isoniazid; RIP: Rifampicin; PIZ: Pirazinamide. HAART: Highly active antiretroviral therapy. 40 cases from United States of America between 1998 and 2006; 28 cases from Spain between 1995-2005; 88 cases from Switzerland between 1996 and 2000; 461 cases from Spain between 1994 and 2004; 29 cases from United States of America between 1999 and 2003; 34 cases from France between 1997 and 2000; 77 cases from Sweden between 1995 and 2005; 31 cases from Asia between 2004 and 2006.

liver transplantation were female^[24]. Thus, age and female gender may affect the clinical course of DILI. As immune responses to drugs and altered drug metabolism are possible mechanisms in DILI pathogenesis, different immune status or drug metabolism according to age or gender may lead to differences in the occurrence of DILI^[25,26]. However, Shapiro and Lewis reported that factors such as age (over 55 years old), gender (female dominant), or the history of alcohol intake were not specific for DILI based on the evaluation of recent DILI cases using the CIOMS/RUCAM scale^[27]. Therefore, risk factors for DILI must be analyzed carefully in future. Moreover, genetic factors for drug metabolism, such as polymorphisms of cytochrome P (CYP) 450 or deficiency of N-acetyltransferase, have been reported to contribute to DILI^[28,29]. Interestingly, a recent report suggested an association between the daily dose of drug ingested and idiosyncratic DILI, and the number of cases and poor outcome of DILI were reported to increase in a dose-dependent manner^[30]. Furthermore, underlying liver disease or systemic viral infection may increase susceptibility to DILI. In particular,

DILI caused by anti-tuberculous therapy is known to be increased in patients with hepatitis B or C virus infection^[31]. Anti-retroviral therapy in HIV infection was reported to induce severe hepatitis and lead to acute liver failure^[32]. The mechanisms by which HIV infection predisposes patients to severe DILI are unknown, but activation or sensitization of the innate immune system by HIV infection may be involved. Moreover, hepatic steatosis in nonalcoholic fatty liver disease (NAFLD) increases the risk of DILI^[33]. Mitochondrial dysfunction or the existence of oxidative stress seen in NAFLD may affect the occurrence and severity of DILI.

Clinical diagnosis of DILI

There are few clinical features associated specifically with DILI. Although fever, rash, arthralgia, and eosinophilia are symptoms and signs of an immunoallergic reaction to a drug, they can also be seen without taking any drugs and the frequencies in patients with DILI are not high. General fatigue, appetite loss, and splenomegaly, often seen in patients with viral hepatitis that may be helpful for differential diagnosis at initial presentation, are also

Table 3 Axes and scores of four representative scales utilized for diagnosis of drug-induced liver injury

NADRPS		CIOMS/RUCAM		M&V		DDW-J	
Axis	Score	Axis	Score	Axis	Score	Axis	Score
Chronological criteria		Chronological criteria		Chronological criteria		Chronological criteria	
Illegibility in onset	-1 to +2	From drug intake until onset	+1 to +2	From drug intake until onset	+1 to +3	From drug intake until onset	+1 to +2
		From drug withdrawal until onset	0 to +1	From drug withdrawal until onset	-3 to +3	From drug withdrawal until onset	0 to +1
Course of the reaction		Course of the reaction		Course of the reaction		Course of the reaction	
	0 to +1	Risk factors Age	-2 to +3		-3 to +3	Risk factors	-2 to +3
		Alcohol (or Pregnancy) ¹	0 to +1			Alcohol (or Pregnancy) ¹	0 to +1
		Concomitant therapy	-3 to 0				
Exclusion of other causes	-1 to +2	Exclusion of other causes	-3 to +2	Exclusion of other causes	-3 to +3	Exclusion of other causes	-3 to +2
		Previous information	0 to +2	Previous information	0 to +2	Previous information	0 to +1
Rechallenge	-1 to +2	Rechallenge	-2 to +3	Rechallenge	0 to +3	Rechallenge	0 to +3
Placebo response	0 to +1						
Drug concentration and monitoring	0 to +1			Extrahepatic manifestations rash, fever, arthralgia, eosinophilia, cytopenia	0 to +3	Extrahepatic manifestations eosinophilia	0 to +1
Dose relationship	0 to +1						
Previous exposure and cross-reactivity	0 to +1						
Any objective evidence	0 to +1					DLST	0 to +2
≥ 9	Definitive	> 8	Definitive	≥ 18	Definitive	≥ 5	Definitive
5 to 8	Probable	6 to 8	Probable	14 to 17	Probable	3 to 4	Probable
1 to 4	Possible	3 to 5	Possible	10 to 13	Possible	≤ 2	Unlikely
≤ 0	Unlikely	1 to 2	Unlikely	6 to 9	Unlikely		
		≤ 0	Excluded	≤ 5	Excluded		

¹Cholestatic/Mixed cases; DLST: Drug lymphocyte stimulation test.

not common in non-fulminant DILI. As there are many causes of liver injury, it is essential to exclude other etiologies in the diagnosis of DILI. Other etiologies include viral hepatitis (hepatitis A virus, hepatitis B virus, hepatitis C virus, hepatitis E virus, EB virus, cytomegalovirus, human herpes virus-6, parvovirus B19, *etc.*), biliary diseases such as cholelithiasis, alcohol abuse, NAFLD, autoimmune liver diseases, and hereditary diseases, such as hemochromatosis, α_1 -antitrypsin deficiency, and Wilson's disease. Among these possible causes of liver injury, diagnosis of acute onset autoimmune hepatitis (AIH) is sometimes difficult, because scores using the International Autoimmune Hepatitis Group scoring system for the diagnosis of AIH, serum IgG levels or antinuclear antibody titers are often low in acute AIH. Histological examination of the liver may be useful for the differential diagnosis. Taken together, a low threshold of suspicion, thorough history including recent and past drug exposure, exclusion of other possible etiologies, or occupational hazards with exposure to potential toxins, are important in making an accurate diagnosis of DILI^[20,34]. Some clinical scales are available for the diagnosis of DILI. However, it is impractical to apply these diagnostic scales for each patient with liver injury taking medications. In addition, most patients take more than one drug, and identification of a single drug as a causative agent is difficult, even in cases where DILI is strongly suspected, using these scales. Moreover, patients with underlying liver or systemic diseases which also affect liver

biochemical tests, complicate the diagnosis of DILI.

Clinical scales available for diagnosis of DILI (Table 3)

As there are no standard criteria for diagnosis of DILI, various clinical scales have been developed. The Naranjo Adverse Drug Reactions Probability Scale (NADRPS) was proposed in 1981 for assessment of adverse drug reactions^[35]. NADRPS has been widely used for DILI due to its simplicity and wide applicability, despite not being developed specifically for diagnosis of DILI. Although simplicity is important for practical use, NADRPS has been reported to have low sensitivity and negative predictive values, and to exhibit a limited capability to distinguish among adjacent categories of probability such as "possible" and "probable"^[36]. In the early 1990s, the diagnostic scale called the Council for International Organizations of Medical Sciences (CIOMS) or Roussel Uclaf Causality Assessment Method (RUCAM), was proposed at the International Consensus Meeting by Danan and Benichou^[4]. It was also called the French method, because Danan had previously reported the diagnostic criteria for acute cytolytic hepatitis in France^[21]. CIOMS/RUCAM is applied for classification of the pattern of liver injury, hepatocellular type, cholestatic type, or mixed type, as described above. This scale is determined by a score based on 7 criteria, including temporal relationship, clinical course (response after withdrawal of drug), risk factors, concomitant drugs, exclusion of other non-drug

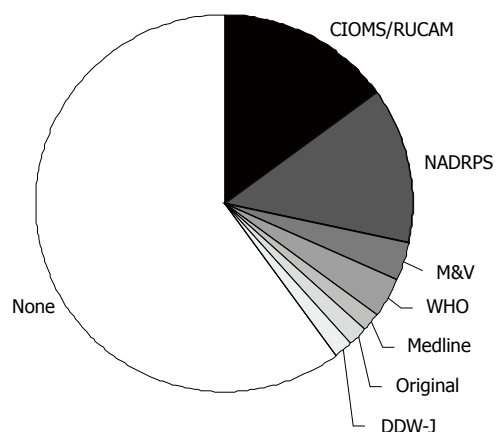


Figure 1 Percentages of methods for causality assessment utilized for diagnosis of DILI, reported during the last decade.

etiologies, likelihood of a reaction based on package labeling, and rechallenge. It has been widely used as a standardized scale with high reliability, reproducibility, and specificity. More recently, Maria and Victorino (M&V) reported a scale called the clinical diagnostic scale (CDS), which simplified the CIOMS/RUCAM using only 5 criteria^[37]. It has often been noted that false negative judgments are often made in cholestatic DILI cases because the pattern of liver injury is not taken into consideration in the M&V scale^[38]. Moreover, DILI cases with long latency periods and evolution to chronic disease after withdrawal (especially cholestatic type) were poorly diagnosed, and there was no agreement in cases of fulminant hepatitis^[39]. The M&V scale emphasizes the immunoallergic reactions, such as extrahepatic manifestations^[40]. In Japan, a diagnostic scale was proposed by reference to the CIOMS/RUCAM scale in Digestive Disease Week Japan (DDW-J) 2004, and includes a drug-lymphocyte stimulation test (DLST) as a diagnostic factor^[41]. The DDW-J scale was reported to have higher sensitivity than the CIOMS/RUCAM (93.8% *vs* 77.8%, respectively) in the analysis of 127 Japanese patients. However, this scale must be evaluated in non-Japanese patients to verify its universal usefulness.

A review of 61 case reports of DILI in the PubMed database over the last decade regarding diagnostic methods used^[42-102] (Figure 1, Table 4) revealed that the CIOMS/RUCAM was the most widely utilized for diagnosis of DILI (10/61 case reports, 16.4%), followed by NADRPS (8/61, 13.1%), M&V (CDS) (2/61, 3.3%), WHO database (2/61, 3.3%), Medline (1/61, 1.6%), Original (1/61, 1.6%), DDW-J (1/61, 1.6%), and none (38/61, 62.3%). The case reports using the WHO database^[88,98] or Medline^[77] based DILI diagnosis on reports of the suspected drug as a causative drug in the database in addition to circumstantial evidence (history of drug intake, onset of liver injury, and exclusion of other causes). In the case of original criteria^[73], DILI diagnosis was made using the following criteria: occurrence of hepatic damage directly related to drug administration, exclusion of other causes of hepatitis, recovery of hepatic function tests after cessation of

drug therapy, and liver histology. Although the CIOMS/RUCAM scale is the most widely used and thus currently seems to be the standard method for diagnosis of DILI, it should be emphasized that many physicians still make a diagnosis of DILI based on their own judgment probably because of the complexity of the scoring systems available.

Additional tests to confirm the diagnosis of DILI and identify a single causative drug

As mentioned above, patients are often taking several drugs only one of which is responsible for liver injury in most cases. However, even when clinical scales for DILI strongly suggest a given drug as a cause of liver injury, identification of the single causative drug cannot be established with these scales. Rechallenge with a potential causative drug to establish a diagnosis is one of the diagnostic methods in the CIOMS/RUCAM criteria^[4,21]; however, it is not advised and may be contraindicated from an ethical viewpoint. As an alternative way to establish the diagnosis of DILI and the identification of a single causative drug, some additional tests using samples from the patient, such as peripheral blood, could be helpful. One of the most commonly used methods is DLST, which is performed as follows^[103]: Lymphocytes collected from the heparinized peripheral blood of patients are incubated with various dilutions of the suspected causative drug. Lymphocyte proliferative response is evaluated by monitoring ³H-thymidine uptake. DLST is widely used in Japan and is incorporated into the diagnostic criteria in Japan (DDW-J scale). However, sensitivity is around 50% and the lymphocyte response to the suspected causative drug may not necessarily be related to liver injury. Another test using peripheral blood of patients is the leukocyte migration test (LMT), which has been reported to be more useful than DLST^[104]. This test involves assaying the chemotaxis of granulocytes from one chamber to another chamber containing mononuclear cells, due to the chemotactic factor produced by the mononuclear cells after incubation with the suspected drug solution. Furthermore, Murata *et al*^[105] reported a cytokine production test as a method to analyze the immunological pathogenesis of DILI, which also showed high sensitivity for diagnosis. In this analysis, HepG2 cells, which reserve the activities of metabolic enzyme such as CYT450, are first incubated with the suspected drug diluents, and the mixtures of the extract and culture medium of HepG2 are then incubated with peripheral blood lymphocytes isolated from the patients. Intracytoplasmic cytokine profiles of the lymphocytes, such as interferon- γ , tumor necrosis factor- α , or interleukin-2, are finally evaluated by flow cytometry. Although these tests are useful methods for the diagnosis or identification of a single causative drug, they are not simple to perform, and may not be feasible for routine examination. However, if a single causative drug cannot be determined, patients may have to avoid several drugs, mostly non-hepatotoxic drugs, for the

Table 4 Diagnostic methods used for diagnosis of drug-induced liver injury during the last decade

Drug	Type ²	Criteria	Country	Yr
Acetaminophen ¹	H	None	Italy	2008
Dexketoprofen trometamol	H	None	Spain	2008
Anabolic-androgenic steroids	C	None	Mexico	2008
Quizalofop-p-ethyl	M	CIOMS/RUCAM	Greece	2007
Amoxicillin/clavulanate	M	None	USA	2007
Fenofibrate	H	None	Poland	2007
INH/RMP/PZA	M	None	USA	2007
Risperidone, Quetiapine	C	NADRPS	USA	2007
Clindamycin	C	NADRPS	Turkey	2007
Bupropion	M	CIOMS/RUCAM, M&V	USA	2007
Flutamide, Cyproterone	H	CIOMS/RUCAM	Spain	2007
Levothyroxine	H	DDW-J	Japan	2007
5-Fluorouracil ¹	H, M	NADRPS	New Zealand	2007
Sairei-to	H	LMT ³	Japan	2007
Terbinafine	H	NADRPS, CIOMS/RUCAM	USA	2007
Ezetimide	H	None	USA	2007
Terbinafine	M	None	USA	2007
Infliximab ¹	H, C	None	Colombia	2007
Methylenedioxymethamphetamine	M	None	Canada	2006
Methylprednisolone	H	NADRPS	Turkey	2006
Shen-min	H	CIOMS/RUCAM	China	2006
Nimesulide	H	None	Italy	2006
Nevirapine	H	None	France	2006
Sirolimus	H	None	Poland	2005
Amiodarone	H	None	Japan	2005
Proguanil, Chloroquine	M	CIOMS/RUCAM	France	2005
Sulpyrine, Clarithromycin	H	None	Japan	2005
Glimepiride	C	None	Greece	2005
Flucloxacillin	M	None	Australia	2005
Sulbactam/ampicillin	C	NADRPS	Turkey	2004
Hydrochlorothiazide	M	NADRPS	Israel	2004
Ketoconazole	M	Original criteria	Korea	2003
Nimesulide	M	None	Turkey	2003
Ramipril	C	None	Canada	2003
Gemcitabine	M	None	USA	2003
Amoxicillin/clavulanate, Ciprofloxacin	H	Medline	USA	2003
Bupropion, Carbimazole	H	NADRPS	Singapore	2003
Ciprofloxacin	H	CIOMS/RUCAM	Germany	2003
6-Thioguanine	H	None	USA	2003
Terfenadine, Oxatamide	M	None	Japan	2002
Pioglitazone	M	None	USA	2002
Danazol	H	None	Japan	2001
Levofloxacin	H	None	USA	2001
Captopril ¹	M	None	Israel	2001
Pioglitazone	H	None	Japan	2001
Celecoxib	M	None	USA	2001
Nimesulide	M	WHO database	Switzerland	2001
Flutamide ¹	H	CIOMS/RUCAM, M&V	Spain	2001
Risperidone	C	None	Germany	2001
Zafirlukast	H	None	USA	2000
Troglitazone	H	None	USA	2000
Stavudine ¹	H	None	USA	2000
Benzazepam ¹	M	None	Spain	2000
Rosiglitazone	H	None	USA	2000
Nitrofurantoin	M	None	Israel	1999
Nimesulide ¹	H, M	CIOMS/RUCAM	Belgium	1998
Omeprazole	H	WHO database	Switzerland	1998
Troglitazone	M	None	USA	1998
Acarbose ¹	H	None	Japan	1998
Benzylpenicillin	H	CIOMS/RUCAM	Switzerland	1997
Terbinafine	M	None	France	1997

¹Cases reported in multiple numbers, not in a single case, are summarized. ²Type of liver injury. H: hepatocellular; C: cholestatic; M: mixed. ³LMT, Lymphocyte migration test.

rest of their lives, seriously limiting treatment of other diseases. Therefore, these tests should be considered in selected cases.

Role of histological examination of the liver for the diagnosis of DILI

The features of liver histology in drug-induced hepatitis

are as follows: (1) demarcated perivenular (acinar zone 3) necrosis; (2) minimal hepatitis with canalicular cholestasis; (3) poorly developed portal inflammatory reaction; (4) abundant neutrophils; (5) abundant eosinophils; and (6) epithelioid-cell granulomas^[106]. However, liver histology in DILI may not be diagnostic in most cases. Moreover, centrilobular necrosis with minimal portal inflammation is relatively characteristic of DILI, but similar histological features can be seen in acute-onset autoimmune hepatitis. Plasma cell infiltration in portal tracts, which is often prominent in autoimmune hepatitis, may be helpful for differential diagnosis in such cases. The major role of histological examination is therefore to exclude other possible causes of liver injury rather than to make a final diagnosis of DILI. Therefore, it is not recommended as a routine or early examination for the diagnosis of DILI.

EARLY MANAGEMENT FOR DILI

As described above, DILI has a wide spectrum of manifestations, ranging from asymptomatic mild biochemical abnormalities to severe hepatitis with jaundice. In most cases of DILI, liver injury would be expected to improve following discontinuation of the drug suspected to be responsible. On the other hand, some DILI patients may even show resolution of liver injury without discontinuation of the drug. Therefore, it should be carefully evaluated whether the suspected drug should be discontinued with adequate consideration of the importance of the medication. However, once liver injury progresses to acute liver failure, this has a high fatality rate without liver transplantation^[107]. Although there are no definitive criteria for cessation of the suspected causative drug, some textbooks suggest that ALT less than $5 \times \text{ULN}$ and no symptoms allow continuation of the suspected drug with close observation, whereas ALT of more than $8 \times \text{ULN}$ indicates the need to discontinue the suspected drug^[108,109]. Another textbook suggests that the suspected drug should be stopped only when abnormalities in serum bilirubin, albumin, or prothrombin time-international normalized ratio (PT-INR) are found in addition to elevated serum ALT^[20]. Zimmerman reported that elevation of transaminase activities in combination with jaundice suggests serious liver injury with fatalities. These findings were discussed at the National Institutes of Health in Bethesda, and are recognized as Hy's rule for monitoring DILI, which states that elevation of liver enzymes (AST or ALT more than $3 \times \text{ULN}$ or ALP more than $1.5 \times \text{ULN}$) in combination with elevated bilirubin (more than $3 \times \text{ULN}$) at any time after starting a new drug may imply serious liver injury and the suspected drug should be stopped^[110]. Two recent studies have shown that hepatocellular liver injury with jaundice is sometimes fatal even if the suspected drug is stopped^[9,10]. On the other hand, a recent study showed that cases fulfilling Hy's rule did not always lead to death from DILI^[18]. As many drugs can induce asymptomatic

elevation of liver enzyme levels without severe hepatotoxicity, mild elevations in transaminases do not always require withdrawal of the causative drug. Based on these observations, the FDA recently proposed draft guidelines (<http://www.fda.gov/cder/guidance/7507dft.htm>) in which ALT greater than $8 \times \text{ULN}$, ALT greater than $5 \times \text{ULN}$ for two weeks, ALT greater than $3 \times \text{ULN}$ in association with serum bilirubin greater than $2 \times \text{ULN}$, more than $1.5 \times \text{PT-INR}$, or symptoms of liver injury should be used to predict severe hepatotoxicity and recommend discontinuing the drug^[2]. Hepatocellular liver injury with severe jaundice should be treated carefully, and requires prompt referral to a center with hepatologists. As mentioned above, severe liver injury and fatality occur in cases of hepatocellular injury with jaundice. On the other hand, cholestatic DILI cases could be observed with continuation of the suspected causative drug, except if symptoms related to liver injury occur, such as jaundice, elevation of serum bilirubin (more than $3 \times \text{ULN}$), or prolongation of PT-INR (more than $1.5 \times \text{ULN}$). There have been no reports of beneficial therapies except the use of N-acetylcysteine for acetaminophen hepatotoxicity. Corticosteroid therapy may be used in DILI cases with evident hypersensitivity, but it does not have proven benefits^[107]. Management of DILI involves prompt withdrawal of the drug suspected to be responsible. A positive de-challenge is a 50% decrease in serum ALT within 8 d of discontinuation of the suspected drug in the hepatocellular type, which is also included in the CIOMS/RUCAM criteria^[5,21]. On the other hand, improvement of biliary enzymes after cessation of the suspected drug usually requires a longer period in cholestatic type. However, the time course after cessation of the suspected drug does not always help in early diagnosis and management of DILI, because some patients should be evaluated promptly and managed as suspected DILI on first presentation.

PROPOSAL OF PRACTICAL GUIDELINES FOR DIAGNOSIS AND EARLY TREATMENT OF DILI

Many drugs can cause abnormalities in liver function tests without any symptoms suggestive of liver disease. Preplanned liver function tests should be performed whenever treatment with a new drug is started. In patients with abnormalities in liver function tests without an obvious cause, a careful history, including not only hospital medications but also herbal remedies or supplements, should first be obtained. History taking should also include drug dosage, administration route, previous administration, any concomitant drugs, alcohol consumption, and underlying chronic liver disease and symptoms such as arthralgia. Moreover, family history of adverse drug reactions may be useful for the diagnosis of DILI. On physical examination, patients should be checked for fever, rash, or jaundice. In particular, jaundice should be evaluated carefully,

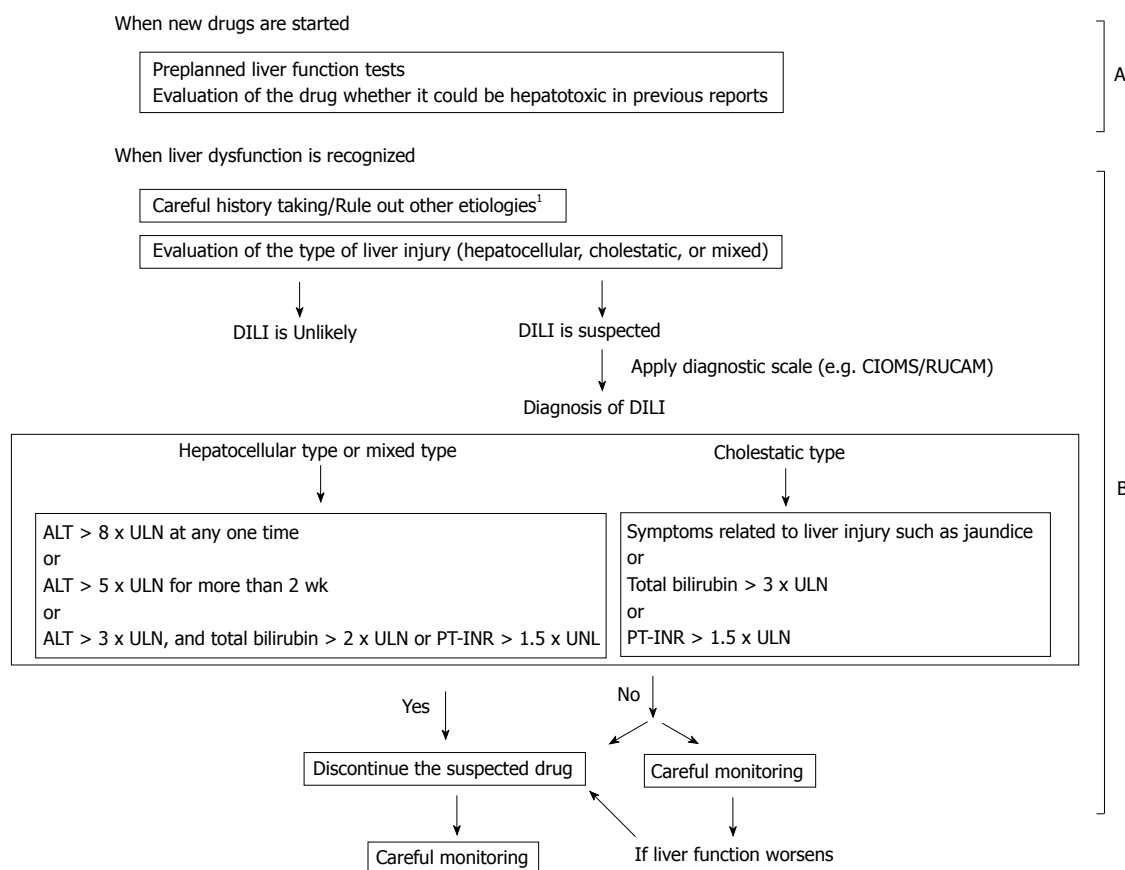


Figure 2 Algorithm for management of DILI. A: When new drugs are started; B: When liver dysfunction is recognized. The type, severity, and causes of liver injury should be assessed promptly. ¹Imaging studies such as ultrasonography should be performed in cases with suspected bile duct disorders.

Table 5 Examinations that should be performed in a patient with suspected DILI

Test	Subjects that can be evaluated
Hematological test ¹ Blood count (including eosinophils)	Determination of the type of liver injury (the ratio of ALT and ALP)
Biochemical test ¹ Aspartate aminotransferase (AST) Alanine aminotransferase (ALT) Lactate dehydrogenase γ -glutamyl transpeptidase (γ -GTP) Alkaline phosphatase (ALP) Total bilirubin (including direct and indirect bilirubin) Albumin Choline esterase (ChE) Total cholesterol (Cho)	Possibility (e.g. Increase in eosinophil count, the existence of mixed type liver injury without any biliary disorders on imaging studies, High IgG level (> 2 g/dL) is suspicious of autoimmune hepatitis. Antibodies against hepatitis virus may be false-negative especially in the early phase of infection. Instead, measurement of viral RNA or DNA may be useful for the diagnosis. HDV (requires concomitant HBV infection) and HEV are relatively rare in advanced countries. Although, liver injury caused by EBV or CMV is also relatively rare, young patients with possible DILI should be checked for EBV or CMV).
Coagulation test ¹ Prothrombin time international ratio (PT-INR)	
Serological test ¹ IgG, IgA, IgM Anti-nuclear antibody (ANA) Anti-mitochondrial antibody (AMA or M2)	Severity (Marked increase or decrease in white blood cell count, decrease in platelet count. Increase in bilirubin level, decrease in albumin, ChE or Cho levels. Decrease in the ratio of direct/total bilirubin (< 0.67). Prolongation of PT-INR).
Viral serology IgM anti-HA ¹ HBsAg ¹ , IgM-HBc ¹ , anti-HBc, HBV-DNA HCV-Ab ¹ , HCV-RNA HDV-Ab, HDV-DNA HEV-Ab, HEV-RNA IgM-EBV IgM-CMV	
Imaging study Ultrasonography (US) ¹	

¹Tests which should be carried out first. Ig: Immunoglobulin; HA: Hepatitis A; HBsAg: Hepatitis B surface antigen; HBc: Hepatitis B core; HBV: Hepatitis B virus; HCV: Hepatitis C virus; Ab: Antibody; HDV: Hepatitis D virus; HEV: Hepatitis E virus; EBV: Epstein-Barr virus; CMV: Cytomegalovirus.

because it is a sign of severe liver injury indicating the necessity for prompt cessation of the suspected drug. Liver function tests including serum transaminase, ALP, γ -glutamyl transpeptidase, and bilirubin, as well as hematological tests including eosinophil count and coagulation tests should be performed. Classification of the pattern of liver injury should be done as early as possible because clinical course, possible etiologies, and causative drugs are different for each pattern^[11]. Other etiologies, such as viral infection, autoimmune liver disease, or biliary disease, should be excluded by serological tests or imaging studies if necessary. DILI cases with severe hepatitis showing elevation of serum bilirubin to more than $3 \times \text{ULN}$ may lead to liver failure, and should be treated carefully with referral to the hepatologist after discontinuing all suspected drugs. The list of recommended tests which should be performed in the diagnosis of DILI in patients with liver injury are shown in Table 5. Although accidental readministration of the causative drug may be beneficial for diagnosis of DILI, it may lead to severe liver injury and may even be fatal, and so is not recommended. Moreover, the probability of DILI should also be evaluated using a diagnostic scoring system, such as the CIOMS/RUCAM criteria. However, there is as yet no gold standard set of diagnostic criteria. The initial treatment usually involves withdrawal of the suspected drug. If the causative drug cannot be discontinued because the patient is receiving many drugs or the underlying disease is serious, medications may be continued with careful monitoring. Additional tests, such as the DLST, LMT, or cytokine production test, may be beneficial to identify the causative drug (Figure 2).

CONCLUSION

The spectrum of DILI is both diverse and complex. Although liver injury is often mild and does not require treatment in these patients, DILI may lead to severe hepatitis with a risk of death. Therefore, adequate initial management after achieving an accurate diagnosis is important for physicians. Although the incidence of DILI is reported to be increasing, the precise frequency is difficult to estimate because of the lack of a worldwide monitoring system and the lack of a gold standard for diagnosis. Establishment of a worldwide network for monitoring the adverse events of drugs and a universal diagnostic system for DILI are important for accurate diagnosis, and may lead to better management of DILI.

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TOPIC HIGHLIGHT

Carlos J Pirola, PhD, FAHA, Series Editor

Dynamic localization of hepatocellular transporters in health and disease

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Abstract

Vesicle-based trafficking of hepatocellular transporters involves delivery of the newly-synthesized carriers from the rough endoplasmic reticulum to either the plasma membrane domain or to an endosomal, submembrane compartment, followed by exocytic targeting to the plasma membrane. Once delivered to the plasma membrane, the transporters usually undergo recycling between the plasma membrane and the endosomal compartment, which usually serves as a reservoir of pre-existing transporters available on demand. The balance between exocytic targeting and endocytic internalization from/to this recycling compartment is therefore a chief determinant of the overall capability of the liver epithelium to secrete bile and to detoxify endo and xenobiotics. Hence, it is a highly regulated process. Impaired regulation of this balance may lead to abnormal localization of these transporters, which results in bile secretory failure due to endocytic internalization of key transporters involved in bile formation. This occurs in several experimental models of hepatocellular cholestasis, and in most human cholestatic liver diseases. This review describes the molecular bases involved in the biology of the dynamic localization of hepatocellular transporters and its regulation, with a focus on the involvement of signaling pathways in this process. Their alterations in different experimental models of cholestasis and in human

cholestatic liver disease are reviewed. In addition, the causes explaining the pathological condition (e.g. disorganization of actin or actin-transporter linkers) and the mediators involved (e.g. activation of cholestatic signaling transduction pathways) are also discussed. Finally, several experimental therapeutic approaches based upon the administration of compounds known to stimulate exocytic insertion of canalicular transporters (e.g. cAMP, tauroursodeoxycholate) are described.

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Key words: Hepatocellular transporters; Cholestasis; cAMP; Bile salts; Vesicular trafficking; Endocytosis; Signaling pathways

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INTRODUCTION

Bile secretion is a highly-regulated process. Such regulation is aimed at coping with the physiological demand for hepatocellular transport of endo- and xenobiotics. This is achieved by modulation of the constitutive expression, dynamic localization or intrinsic activity of relevant transport systems located at the sinusoidal (basolateral) and canalicular (apical) membranes of the hepatocyte.

Modulation of carrier transport activity may occur at different time scales. Long-term regulations occur by changes in carrier turnover, which leads to modification of the synthesis-degradation balance. Altered synthesis rate involves transcriptional or translational changes in carrier expression. On the other hand, modification of the carrier degradation rate is a post-translational

process. This latter event may involve, as an initiating step, sustained internalization of the carrier protein from its plasma membrane domain, followed by lysosomal breakdown.

In contrast to this irreversible fate, transitory, reversible changes in transporter localization by vesicle-mediated insertion/internalization from/to an endosomal recycling compartment may occur as part of a short-term, physiological mechanism aimed at quickly modulating carrier density at the plasma membrane. This is a tightly regulated process, and the signaling mediators involved are being actively characterized.

Apart from its role in biliary physiology, changes in the proper localization of hepatocellular carriers also occur in a number of pathological conditions, and they may partly explain the cholestatic manifestations in these liver diseases. This has encouraged investigators to better understand the mechanisms involved in this particular pathomechanism at a molecular level, and to envisage and test in experimental models of cholestasis new therapeutic approaches based upon its prevention.

This article aims to give an overview of this subject, by summarizing the current information available in the literature on physiological regulation and cholestatic changes in hepatocellular carrier dynamic localization, as well as its beneficial modulation by therapeutic agents.

HEPATOCELLULAR TRANSPORT SYSTEMS

The hepatocyte is a polarized cell that expresses differential transport systems in its plasma membrane domains. These transporters play a key role in the vectorial transfer of solutes and water from sinusoidal blood into bile, thus contributing to bile formation and the biliary excretion of many xenobiotics. Most of these transport proteins have been identified by molecular cloning, and their transport properties characterized by functional studies. Their localization and transport function are shown in Figure 1.

Sinusoidal solute uptake transporters

Liver sinusoids possess a specific architecture that allows passage of organic compounds bound to albumin through endothelial fenestrae into the space of Disse, from where they can be taken up by the sinusoidal transport systems of the hepatocytes^[1].

Basolateral uptake transporters can be divided into Na^+ -dependent and Na^+ -independent systems. Na^+ -dependent uptake involves co-transport of solutes with Na^+ , and is driven by the electrochemical Na^+ gradient generated and maintained by the Na^+/K^+ -ATPase, which is strategically localized at the sinusoidal membrane. The Na^+ -independent transport of organic anions is driven primarily by anion exchange.

Bile salts are the predominant organic solutes in bile, and the main determinants of bile flow^[2]. Bile salts are mainly taken up by the Na^+ /taurocholate co-transporting polypeptide (NTCP/Ntcp for humans

and rodents, respectively; also known as SLC10A1/Slc10a1)^[3]. A remaining fraction is taken up by a Na^+ -independent transport system mediated by the organic anion-transporting polypeptide (OATP/Oatp) family of transporters^[4,5]. In addition to conjugated and unconjugated bile salts, Oatps/OATPs accept other cholephilic compounds, including glucuronidated (and maybe unconjugated) bilirubin, exogenous organic anions (e.g. sulphobromophthalein), leukotrienes, estrogen-conjugates (e.g. estrone-3-sulfate or estradiol-17- β -d-glucuronide), thyroid hormones, mycotoxins, and numerous xenobiotics^[3,6-8]. Four OATPs have been cloned and characterized from human liver, namely: OATP1A2 (SLCO1A2/SLC21A3; formerly, OATP-A), OATP1B1 (SLC21A6; formerly, OATP-C or LST-1), OATP1B3 (SLC21A8; formerly, OATP-8) and OATP2B1 (SLC21A9; formerly, OATP-B). There are three Oatps identified in rats, namely: Oatp1a1 (*Slc21a1*; formerly, Oatp1), Oatp1a4 (*Slc21a5*; formerly, Oatp2) and Oatp1b2 (*Slc21a10*; formerly, Oatp4 or Lst-1). Oatp1b2 is the rodent ortholog of both OATP1B1 and OATP1B3^[9].

Hepatocellular uptake of organic cations is mediated by two separate transport systems, which depends on the substrate molecular size^[10]. Thus, small (type I) organic cations are taken up by the organic cation transporter, OCT1/Oct1 (SLC22A1/Slc22a1), which is electrogenic in nature. On the other hand, human OATP-A (but not the remaining members of the OATP family) and rat Oatp2 mediate the uptake of bulky (type II) organic cations.

Canalicular solute export transporters

After traversing the cell by Fick's diffusion, mostly bound to high-affinity cytosolic proteins, cholephilic compounds are excreted into bile mainly by ATP-dependent pumps of the superfamily of ATP-binding cassette (ABC) transporters, in particular those belonging to the family of multidrug-resistance proteins, MDR/Mdr, or to the family of multidrug-resistance-associated proteins, MRP/Mrp.

MDRs/Mdrs are members of the ABC superfamily that were originally described in cancer cell lines, where they confer resistance to therapeutic agents. Three gene products were identified in rodents, Mdr1a (Abcb1a), Mdr1b (Abcb1b) and Mdr2 (Abcb4), and two in humans, MDR1 (ABCB1) and MDR 3 (ABCB4). MDR1/Mdr1 functions as an efflux pump for a wide range of amphiphilic, bulky type II cationic drugs, together with other hydrophobic compounds, such as endogenous and exogenous metabolites or toxins, steroid hormones, hydrophobic peptides and even glycolipids^[8]. Two closely related but functionally distinct Mdr1 isoforms, mdr1a and mdr1b are present in the murine but not in the human phenotype^[11]. MDR3/Mdr2 functions as a flippase, which translocates phosphatidylcholine (PC) from the inner to the outer leaflet of the canalicular membrane, followed by release of PC-containing vesicles from the outer leaflet into bile, a process facilitated by the detergent properties of luminal bile salts^[12].

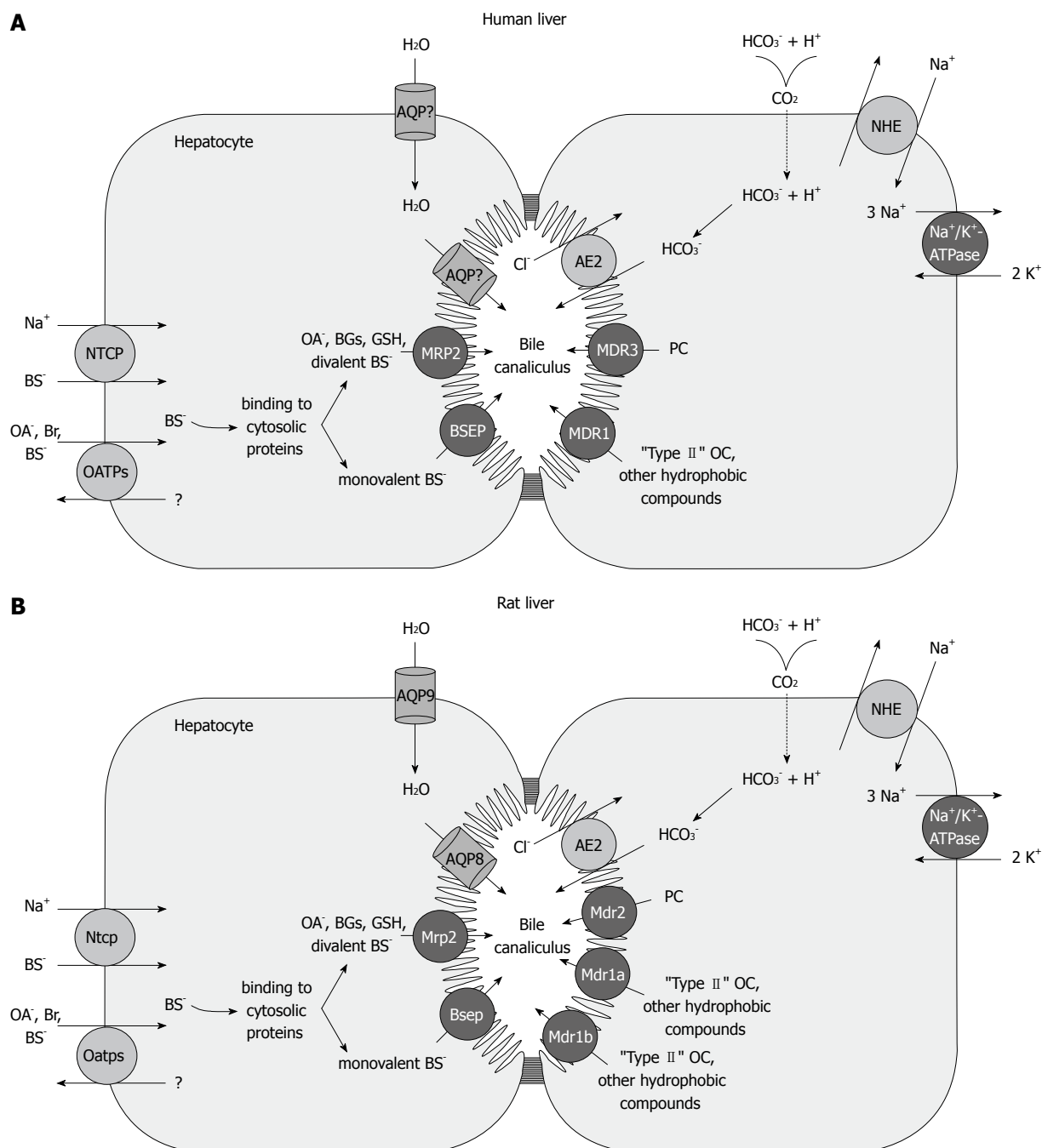


Figure 1 Localization and function of sinusoidal and canalicular hepatocellular transporters. A: humans; B: rodents. The Na⁺-dependent sinusoidal uptake of bile salts is mediated by NTCP (human)/Ntcp (rat). The Na⁺-independent hepatic uptake of organic anions (OA⁻), Bile salts and type II organic cations (OC⁺) is mediated by members of the OATP/Oatp family. Sinusoidal uptake of type I OC⁺ is mediated by OCT1/Oct1. Transport across the canalicular membrane is driven mainly by ATP-dependent export pumps (black circles). MDR1/Mdr1a, Mdr1b mediates canalicular excretion of amphiphilic type II OC⁺ and other hydrophobic compounds. MDR3/Mdr2 functions as a phosphatidylcholine (PC) flippase. BSEP/Bsep mediates apical excretion of BSs. MRP2/Mrp2 transports non-bile-salt organic anions, such as bilirubin glucuronides, GSH, and sulfated/glucuronidated bile salts. Canalicular transport of HCO₃⁻ is mediated by the Cl⁻/HCO₃⁻ exchanger AE2/Ae2. Aquaporins AQP9 and AQP8 are involved in the transport of water across the rat sinusoidal and the canalicular membrane, respectively. The nature of the water channels in human liver has yet to be characterized.

Monoanionic bile salts are excreted in the canalicular pole by the *bile salt export pump* (BSEP/Bsep; ABCB11/abcb11), another member of the MDR family^[13]. In contrast, canalicular efflux of divalent, bipolar sulfated or glucuronidated bile salts is mediated by the multidrug-resistance-associated protein 2 (MRP2/Mrp2; ABCC2/Abcc2)^[4,14]. This carrier is also engaged in the biliary excretion of many other organic anions, including glutathione S-conjugates (e.g. of leukotriene C4 or

sulphobromophthalein, among others), glucuronides (e.g. of bilirubin and estrogens), and reduced (GSH) and oxidized glutathione (GSSG), the former with low affinity^[15,16]. Both GSSG and GSH are major determinants of the so-called "canalicular bile-salt-independent bile flow"^[17].

The canalicular membrane domain also contains the electroneutral anion exchanger 2 (AE2/Ae2; SLC4A2/slc4a2), which extrudes HCO₃⁻ by exchanging the anion

for biliary Cl^{-} ^[18]. It functions to regulate intracellular pH when hepatocytes are exposed to an alkaline load^[18]. In addition, AE2/Ae2 plays a role in bile flow generation, since HCO_3^- excretion is thought to be an additional primary driving force of the canalicular bile-salt-independent bile flow^[18,19]. Both in humans and rats, three transcript variants of AE2/Ae2 have been described, namely the full-length transcript AE2a/Ae2a, expressed from the upstream promoter in most tissues, and the alternative transcripts AE2b₁/Ae2b₁ and AE2b₂/Ae2b₂, expressed in a more tissue-restricted fashion (mainly in liver and kidney). AE2b_{1/2}/Ae2b_{1/2} transcription is driven from overlapping promoter sequences within intron 2, which result in AE2/Ae2 protein isoforms with short N-terminal differences^[20,21].

Water transporters

For a solute to drive blood-to-bile vectorial water transport primarily, resultant osmotic forces need to be associated with aquaporin (AQP)-mediated transcellular movement of water molecules from plasma to the bile canaliculus. Both immunochemical and functional studies have demonstrated the constitutive expression of the water channel AQP9 at the basolateral membrane of rat hepatocytes, and the regulated expression of the water channel AQP8 at the hepatocellular canalicular membrane domain^[22-24]. As a result of it being inserted in the canalicular membrane on demand, AQP8 is suggested to play a role in bile formation, facilitating the osmotic movement of water under a choleretic stimulus^[23,24]. AQP isoforms that mediate polarized water transport in human hepatocytes, if any, remain to be identified.

MECHANISMS OF NORMAL TRAFFICKING OF HEPATOCELLULAR TRANSPORTERS AND ITS REGULATION BY SIGNALING PATHWAYS

Basolateral transporters

NTCP/Ntcp: Basolateral targeting of NTCP is mediated by a sorting pathway that involves translocation of the protein from the endoplasmic reticulum (ER) to the Golgi apparatus, and from there to the plasma membrane, by a trans-Golgi-network-independent pathway^[25]. The process may also involve microtubular and microfilamental motor proteins. A role for the cytoskeleton in NTCP translocation has been studied in detail using green fluorescent protein (GFP)-tagged NTCP expressed in the HepG2 cell line^[26]. This study showed that targeting of NTCP to the plasma membrane consists of two steps: (1) delivery of NTCP to the region of the plasma membrane *via* microtubules, and (2) insertion of NTCP into the plasma membrane, by a microfilament-mediated mechanism; this actin requirement was also observed in isolated rat hepatocytes^[27]. The latter step more likely involves targeting of NTCP from an early (recycling) endosomal

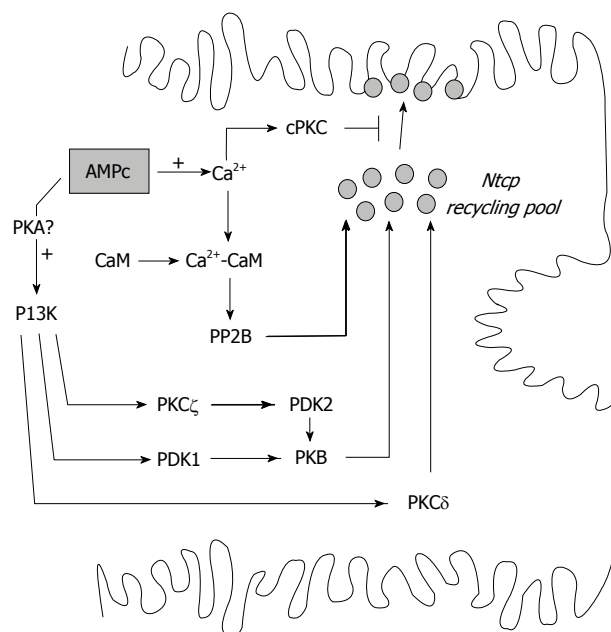


Figure 2 Signaling pathways that regulate the cAMP-induced exocytic insertion of Ntcp into the basolateral membrane. cAMP stimulatory effect involves elevations in cytosolic Ca^{2+} and activation of PI3K-dependent pathway, probably via protein kinase A (PKA). CaM complex activates phosphatase 2B (PP2B), which promotes insertion of Ntcp by dephosphorylation. This pathway is counter-regulated by cPKC. cAMP also stimulates Ntcp targeting by PI3K-dependent activation of PDK1 and subsequent PKB activation. Alternatively, PKB is activated by the concerted action of the atypical PKC ζ and PDK2. Finally, cAMP/PI3K signaling stimulatory pathway may involve PKC δ .

compartment^[28]. These NTCP/Ntcp-containing vesicles also express the microtubule-based motor proteins dynein and kinesin, and the actin-based motor myosin IIa^[28]. This compartment may serve as a reservoir of transporters for their rapid insertion into the sinusoidal membrane under a physiological stimulus that requires their function. It is therefore not surprising that recycling of NTCP/Ntcp from this compartment is a highly regulated process.

The cAMP-elevating hormone glucagon and the permeant cAMP analog dibutyryl cAMP stimulate hepatocyte Ntcp maximal transport in rats by insertional exocytosis from intracellular vesicles that contain the transporter^[29]. The signaling pathways evoked by cAMP that account for this stimulatory effect are depicted in Figure 2. Protein kinase A (PKA) activation^[30], phosphatidylinositol 3-kinase (PI3K) activation^[27,31] and elevations of cytosolic Ca^{2+} ^[30] all mediate the cAMP effect. Although the mechanism of PI3K activation by cAMP has not been elucidated as yet, there is evidence in other cell lines that the cAMP-dependent PKA can activate PI3K by phosphorylation of the PI3K regulatory subunit, p85^[32]; if this applies to hepatocytes, this would explain the dual mediation of PKA and PI3K in the cAMP-stimulatory effect. The downstream mediators of the cAMP-PI3K signaling pathway are under debate, and may be multifactorial. The PI3K downstream enzyme, protein kinase B (PKB, also known as Akt), has been implicated^[27,31]. Coincidentally, hepatocellular swelling, which also evokes the PI3K/

PKB signaling pathway, favors Ntcp translocation to the plasma membrane as well^[31,33]. The effect of PI3K/PKB on Ntcp translocation seems to be mediated by the PI3K-dependent activation of atypical protein kinase C zeta (PKC ζ)^[34]. PKC ζ is downstream of PI3K, since PI3K products activate this PKC isoform^[35,36]. The requirement of PKC ζ for the PKB effect can be explained by PKC ζ modulation of activators upstream of PKB. Activation of PKB requires phosphorylation by 3-phosphoinositide phosphate-dependent kinase 1 (PDK1), followed by phosphorylation by a second kinase, PDK2; this latter kinase phosphorylates and activates PKB fully only when associated with PKC ζ ^[36,37]. In addition, a direct, non-PKB-mediated stimulatory role for PKC ζ on Ntcp translocation has been suggested^[34]. Apart from PKC ζ , cAMP-stimulated PI3K phosphorylates the novel protein kinase C delta (PKC δ) at Thr-505, and the resulting activation seems to be involved in Ntcp membrane translocation as well^[38]. The molecular target/s phosphorylated by PKB, PKC ζ and PKC δ that ultimately account for the translocation of Ntcp are unknown. Ntcp itself seems not to be a target, since cAMP may promote dephosphorylation rather than phosphorylation of the carrier^[39-41]. However, studies in transfected COS-7 and Madin-Darby canine kidney (MDCK) cells using GFP-fused Ntcp constructs that lack the cytoplasmic Ntcp tail, which serves as a signal for basolateral sorting, have demonstrated that this moiety has regulatory phosphorylation sites that are essential for cAMP-induced stimulation of Ntcp translocation^[42]. The relevance of this finding needs to be tested in a more physiological context. Other possible phosphorylation targets, at least of PKC ζ , are the microtubule motors that drive movement of Ntcp-containing vesicles. A majority (75%) of intracellular vesicles containing Ntcp were found to co-localize with PKC ζ in rat hepatocytes, and the motility of these vesicles on microtubules, when assessed using an *in vitro* motility assay, was impaired by both PI3K and PKC ζ inhibitors, and stimulated by PI3K products^[28].

Apart from activating PKA and PI3K, cAMP induces elevations of cytosolic Ca²⁺ in hepatocytes^[43,44]. The subsequent formation of the Ca²⁺-calmodulin (CaM) complex influences Ntcp localization by activating the Ca²⁺/CaM-dependent serine-threonine phosphatase PP2B (also known as calcineurin)^[39]. cAMP promotes both serine and threonine dephosphorylation of Ntcp *via* PP2B^[39-41], and dephosphorylated Ntcp is located preferentially in the plasma membrane^[45]. Phosphorylated Ser-226 in the third cytoplasmic loop of Ntcp may be the target for cAMP-stimulated dephosphorylation^[45]. This cAMP-dependent, Ca²⁺-mediated pathway may be counter-regulated by activation of "classical" (Ca²⁺-dependent) PKC (cPKC), since pan-specific activation of PKC with phorbol esters counteracts the cAMP-stimulatory effect^[30].

OATP/Oatp: Unlike Ntcp, this family of transporters is not stored in intracellular vesicular compartments, and therefore regulation by trafficking is limited to

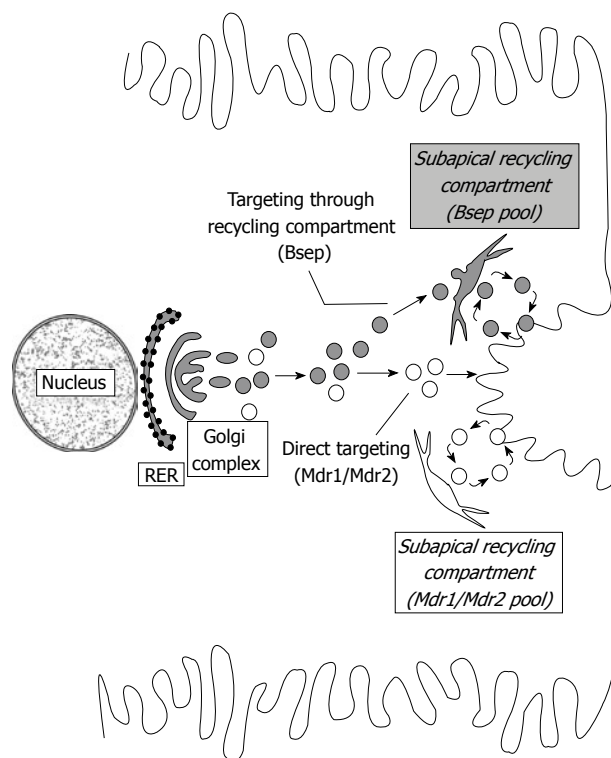


Figure 3 Routes involved in trafficking of canalicular transporters. The trafficking of vesicles delivering Bsep (gray vesicles) or Mdr1/Mdr2 (white vesicles) from the site of synthesis to the canalicular domain is distinct. Mdr1 and Mdr2 are directly targeted to the canalicular membrane, whereas Bsep is indirectly targeted via a subapical, endosomal compartment, which allows the recycling of transporters (exocytic insertion/endocytic internalization). Once targeted, Mdr1 and Mdr2 are also able to recycle between the subapical compartment and the canalicular membrane.

modulation of its transfer from synthesis sites. Sorting of human OATP-C to the basolateral membrane is mediated by both the Golgi complex- and the vacuolar H⁺-ATPase vesicle-mediated membrane sorting pathways, and cAMP positively regulates the first sorting mechanism *via* activation of PKA^[46].

Canalicular transporters

ABC canalicular transporters: Vesicle-based trafficking steps of canalicular export pumps are depicted in Figure 3. Once synthesized by the rough ER, *de novo* ABC canalicular transporters belonging to either the MRP or the MDR family traffic *via* the Golgi complex directly to the apical membrane^[47-49]. Pulse-chase studies using ³⁵S-methionine followed by immunoprecipitation of the ABC transporters from subcellular fractions have revealed that these transporters are targeted directly to the canalicular membrane, as at no time between passage through Golgi and arrival at the canalicular membrane are the ABC transporters localized at the sinusoidal membrane^[49]. However, the post-Golgi trafficking differs among the ABC transporters studied. Mdr1 and Mdr2 are fully delivered to the canalicular membrane 30 min after ³⁵S-methionine administration^[49]. This finding was confirmed for Mdr1 in WIF-B cells, a hybrid of rat hepatoma cells and human fibroblasts that has functional bile canaliculi^[50]. Contrarily, Bsep only reaches

the canalicular membrane after 2 h, which suggests that, unlike Mdr1/2, Bsep is retained in an intracellular endosomal pool prior to delivery to the canalicular membrane^[48]. This intrahepatic, large vesicular pool also serves as a reservoir of ABC transporters, which can be quickly recruited to the canalicular membrane on physiological demand that requires their function (e.g. increased biliary excretion of bile salts for lipid digestion/absorption during the post-prandial period). The recycling process involves exocytic insertion, followed by endocytic internalization once demand is satisfied^[47,48].

Compelling evidence in the literature further supports the existence of this recycling compartment for canalicular hepatocellular transporters. Immunogold electron microscopy studies of rat hepatocytes have revealed that distribution of Bsep is not restricted to the canalicular membrane, but is also detected in electron-translucent vacuolar structures close to the apical, but not the basolateral membrane^[51]. Pericanalicular localization of Mrp2, Bsep and Mdr1 has also been demonstrated by immunofluorescent staining in isolated rat hepatocyte couplets^[52]. Finally, direct visualization of the recycling between the canalicular membrane and subapical endosomes has been observed for Bsep-GDP chimeras in WIF-B cells stably transfected with adenoviral Bsep-GFP constructs^[53]. Chimeric Bsep co-localizes with the marker of recycling endosomes Rab11, and its recycling was microtubule- and microfilament-dependent in both ways^[53]. On the contrary, and unlike the *de novo* transporter pathway, this recycling does not involve the Golgi complex, since it is unaffected by brefeldin A. This suggests that recycling represents an independent step in the whole trafficking of *de novo* ABC transporters to the canalicular membrane, and that only replenishment of this recycling compartment with newly-synthesized transporters is Golgi-dependent.

This large-range, Golgi-dependent vesicular trafficking of ABC transporters has been characterized by our group and others using the couplet model. Sorting of Mrp2 to the apical membrane has been analyzed by studying the spontaneous retargeting of the transporter after Mrp2 internalization that occurs during the isolation process^[54,55]; this vesicle-based trafficking shares the route of newly-synthesized, apically-directed proteins, since it is sensitive to disruption of the Golgi complex function with brefeldin A^[55]. Inhibitors of microtubule polymerization diminish, but do not completely block, the restoration of Mrp2 localization^[54,55]. Re-establishment of hepatocyte couplet secretory polarity is instead strikingly dependent on microfilament organization^[55]. A similar differential cytoskeletal dependency has been suggested to occur for Bsep, as inferred by functional studies upon restoration of the hepatocyte couplet capability to secrete apically the Bsep substrate, cholyl-lysylfluorescein (CLF), and also for $\text{Ca}^{2+}/\text{Mg}^{2+}$ -ATPase, another canalicular transporter^[56]. The vesicle motor protein myosin-II may be crucially involved in the actin-dependent

targeting of Bsep. Co-immunoprecipitation studies have identified myosin-II regulatory light chain as a binding partner of BSEP, and reduced expression of this protein in dominant negative mutant MDCK cells reduces apical membrane BSEP levels^[57]. Furthermore, pharmacological inhibition of myosin II impedes delivery of newly synthesized transporter to the apical membrane in these cells^[57]. These findings suggest that myosin-II is required for BSEP trafficking to the apical membrane in polarized epithelial cells.

Trafficking of ABC transporters from their place of synthesis to the canalicular membrane is under signaling modulation. Studies using the re-polarization approach in hepatocyte couplets described above have shown that the spontaneous canalicular targeting of Mrp2 after isolation and culture is Ca^{2+} - but not PKA-dependent^[55]. The Ca^{2+} -elevating compound thapsigargin (an inhibitor of the ER Ca^{2+} -ATPase) accelerates, whereas the intracellular Ca^{2+} chelator BAPTA/AM and the CaM inhibitor W7 greatly inhibit this process, which suggests Ca^{2+} -CaM dependency. On the other hand, the PKC-dependent signaling pathway is inhibitory in nature, since the PKC activator phorbol 12,13-dibutyrate inhibits this process, whereas both the pan-specific PKC inhibitor staurosporine and the specific inhibitor cPKC Gö6976 accelerate this process. This indicates that, under basal conditions, cPKC exerts an inhibitory effect on long-range trafficking of ABC transporters to the canalicular pole and that the stimulation induced by Ca^{2+} elevations may generate its own counter-regulatory mechanism, by activating cPKC. In this connection, selective activation of cPKC by administration of thymeleatoxin is associated with retrieval of Bsep and loss of bile salt secretory function in isolated rat perfused liver^[58].

Both Roelofsen *et al.*^[54] and our group^[55] have analyzed the influence of cAMP on the time-dependent re-targeting of Mrp2 after isolation-induced Mrp2 internalization. cAMP stimulates this process. This phenomenon is partially inhibited by inhibitors of microtubule polymerization. We have further examined this phenomenon by analyzing the involvement of signaling molecules downstream of cAMP, the cross talk with other signaling pathways, and the dependency of cAMP stimulus on cytoskeleton organization^[55] (Figure 4). The cAMP-sensitive stimulatory pathway shares most downstream signaling constituents with the basal, spontaneous pathway described above, i.e. it is not PKA-dependent, but Ca^{2+} -dependent, *via* Ca^{2+} -CaM complex formation. This cAMP-dependent pathway is also counter-regulated by activation of cPKC^[55]. Interestingly, a similar counter-regulatory cross-talk between cAMP- and PKC-dependent signaling pathways applies to the trafficking of other transporters, including Ae2^[59] and Ntcp^[30]. Another candidate to mediate cAMP-stimulatory effects is PI3K. Studies *in vivo* have revealed that cAMP-mediated stimulation of ABC transporter insertion is inhibited by the PI3K inhibitor wortmannin, and restored by phosphoinositide PI3K products^[60]. PKC δ has been identified recently

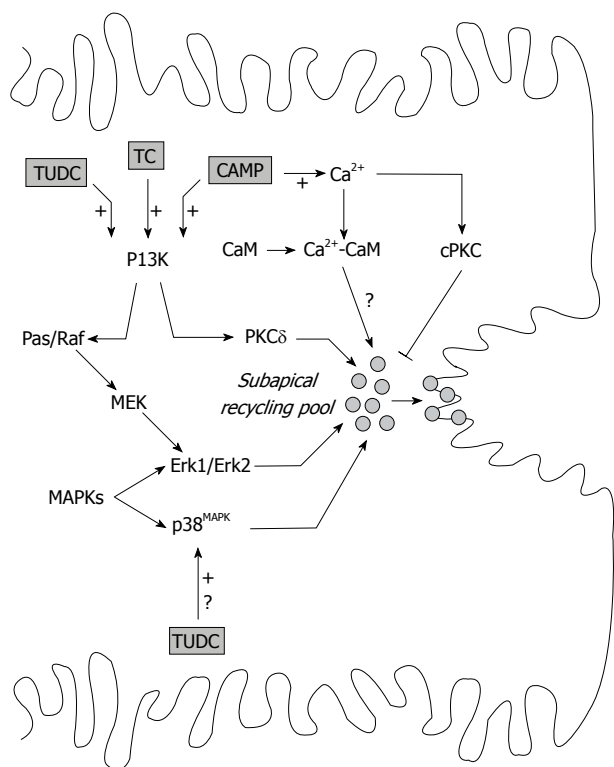


Figure 4 Signaling pathways involved in the exocytic insertion of canalicular transporters promoted by cAMP and by TC and TUDC. cAMP effect involves elevation in cytosolic Ca^{2+} and activation of the PI3K-dependent pathway. Formation of the CaM complex promotes apical insertion of transporters via unidentified mediators, and is counter-regulated by activation of cPKC. PI3K promotes exocytic insertion of canalicular transporters by activation of PKC δ and Erk-1 and Erk-2 of MAPK, via the Ras/Raf- MAPK kinase (MEK)-Erk-1/2 pathway. TC and TUDC also evoke the PI3K-dependent signaling pathway and promote insertion of canalicular transporters via the Ras/Raf-MEK-Erk-1/2 pathway. TUDC also stimulates canalicular carrier insertion by activation of MAPKs of the p38MAPK type, by an unknown mechanism.

as a possible effector of the cAMP-dependent, PI3K-mediated pathways that leads to Mrp2 insertion^[38]. The endogenous bile salt taurocholate (TC), which, as does cAMP, evokes the PI3K-dependent signaling pathway^[61] and activates PKC δ ^[62], also promotes insertion of ABC transporters into the canalicular membrane in a PI3K-sensitive manner^[61].

Another bile salt that stimulates exocytic insertion of canalicular transporters is tauroursodeoxycholate (TUDC)^[63], but its action mechanism seems to involve another set of signaling molecules (Figure 4). TUDC activates within minutes mitogen-activated protein kinases (MAPKs) of both the p38^{MAPK} type^[63] and of the extracellular signal-regulated kinase (Erk) type (Erk-1 and Erk-2)^[64]. These effects are causally linked to increased biliary excretion of Bsep; the latter event having been demonstrated only for p38^{MAPK}^[63]. The stimulus induced by TUDC on Erk-1/2, but not on p38^{MAPK}, is dependent on the sequential activation of PI3K and Ras/Raf^[65]. The two MAPK-dependent pathways seem to act in parallel, and dual activation is required^[63]. Studies in human hepatoblastoma HepG2 cells and in rat hepatocytes have shown that TUDC-stimulated insertion

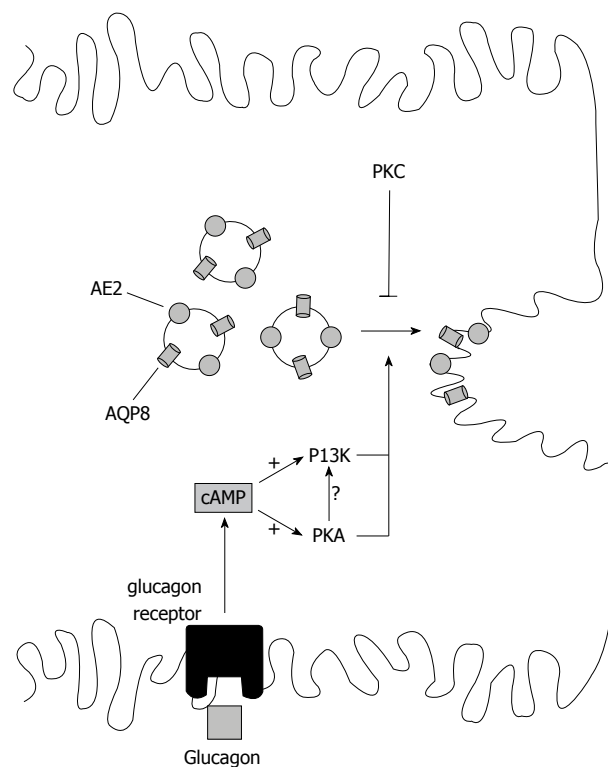


Figure 5 Signaling pathways involved in the co-stimulation of the canalicular targeting of AE2 and AQP8 by cAMP. AE2 and AQP8 are co-localized in the same population of pericanalicular vesicles, thus explaining common signaling modulation. cAMP stimulates AE2 and AQP8 targeting via activation of PKA. The PI3K pathway mediates the cAMP-stimulated, PKA-dependent targeting of AQP8, and probably that of AE2. cAMP effect on both transporters is counteracted by activation of PKC.

of BSEP involves not only increased targeting from the subapical compartment to the canalicular membrane, but also enhanced trafficking from the Golgi complex to the subapical compartment, and that p38^{MAPK} may be a key signaling molecule in mediating this latter effect^[66]. Coincidentally, hypo-osmotic cell swelling, which shares with TUDC several downstream signaling effectors, also stimulates bile salt excretion by activation of Erk-1/2 and p38^{MAPK}^[67], and both types of MAPKs are involved in hypotonicity-stimulated, microtubule-sensitive bile salt excretion^[68,69].

AE2/Ae2: Apart from its functional localization in the canalicular membrane, the canalicular $\text{Cl}^-/\text{HCO}_3^-$ exchanger AE2/Ae2 is present in pericanalicular vesicles^[21,70], which migrate to the canalicular membrane on demand (Figure 5). The sorting in polarized liver cells of the three Ae2 variants, Ae2a, Ae2b₁ and Ae2b₂, has been studied using collagen-sandwiched primary rat hepatocytes^[21]. After 72-96 h, GFP constructs from each recombinant Ae2 isoform co-localize in the canalicular membrane and in subapical, vesicular structures, and no signal is detected at the basolateral pole. This shared sorting of Ae2 isoforms is sensitive to the microtubule-disrupting agent colchicine, which suggests microtubule-dependent vesicular transport and exocytotic insertion of these transporter isoforms in the canalicular membrane.

Microtubule-dependence of Ae2 trafficking has been confirmed by functional studies. Ae2-mediated $\text{Cl}^-/\text{HCO}_3^-$ exchange is increased in rat hepatocytes exposed to a bicarbonate-containing medium or in response to cAMP, and this increased activity is blocked with colchicine^[59]. The cAMP-elevating hormone glucagon also stimulates this activity through a microtubule- and a cAMP-dependent, PKA-mediated mechanism^[71]. The stimulation of $\text{Cl}^-/\text{HCO}_3^-$ exchange activity by cAMP or glucagon is inhibited by PKC agonists^[59,71], which suggests the existence of a counter-regulatory mechanism similar to that occurring for the targeting of Ntcp and ABC canalicular transporters (see above).

AQP8: This water canalicular channel is largely localized in intracellular vesicles in hepatocytes, as demonstrated by both subcellular fractionation^[23], confocal immunofluorescence^[23] and immunoelectron microscopy studies^[72]. As a result of this property, it can be quickly inserted in the canalicular membrane on demand^[24,73]. The cell-permeable cAMP analog dibutyryl cAMP induces redistribution of AQP8 to the canalicular membrane, and increases hepatocyte membrane water permeability in a microtubule-dependent manner^[22,23]. Further studies in isolated rat hepatocytes^[74] have shown that, as with AE2, AQP8 is inserted in the canalicular membrane by the cAMP-elevating hormone glucagon, by a process that involves both PKA and PI3K activation^[75]. Immunofluorescent co-staining studies in WIF-B cells have shown intracellular co-localization of AQP8 and AE2, which suggests that these transporters are expressed in the same population of pericanalicular vesicles^[76] (Figure 5). This explains the similar behavior of both transporters in response to a similar regulatory stimulus. Thus, apart from modulating the biliary secretion of osmotically-active solutes to the bile canaliculus *via* exocytic insertion of relevant carriers (e.g., BSEP, MRP2, AE2), hepatocytes can also modulate their canalicular membrane water permeability by inserting AQP8, thus facilitating the osmotic movement of water under choleretic stimulus.

ALTERATIONS OF THE DYNAMIC LOCALIZATION OF TRANSPORTERS IN LIVER DISEASE

Endocytic internalization of hepatocellular transporters is a common feature in liver disease. This applies mainly to those liver diseases that involve primary impairment in the capability of hepatocytes to produce bile (hepatocellular cholestasis). In these cases, changes in transporter localization may become a major pathomechanism that explains the secretory failure. Alternatively, changes in carrier localization can occur as a secondary consequence of a cholestatic manifestation caused by mechanical impediments to deliver bile to the duodenum (obstructive cholestasis). In this case, transporter mis-localization may aggravate/perpetuate the primary secretory halt. We summarize here the current evidence in the literature that alterations in the dynamic

localization of transporters occur in experimental and human cholestatic liver disease.

Endocytic internalization of transporters in animal models of cholestasis

Endocytic internalization of the main canalicular transporters was first described in experimental models of cholestasis in rodents. Internalization of Mrp2 and Bsep into intracellular vesicles, mainly at the pericanalicular domain, has been shown to occur in experimental models of both obstructive and hepatocellular cholestasis.

Bile duct ligation (BDL): Experimental ligation of the common bile duct in the rat is an accepted model of obstructive cholestasis. BDL leads to a marked alteration in the pattern of staining of both Mrp2 and Bsep, as detected by indirect immunofluorescence microscopy. Paulusma *et al*^[77] have found that, 48 h after BDL in rats, immunostaining of these transporters at the canalicular level becomes fuzzy, contrasting with the well-delimited detection in sham-operated controls. The authors have assumed that this represents mis-localization of the transporters to intracellular vesicles at a subapical compartment, next to the canaliculus. These alterations are accompanied by a severe impairment of the biliary excretion of model solutes. For example, Mrp2-mediated transport of the model substrate dinitrophenyl glutathione is substantially impaired in isolated hepatocytes from rats with BDL^[77]. Endocytic internalization seems not to be circumscribed to Mrp2 or Bsep, as a similar phenomenon was observed for the canalicular enzymes dipeptidyl peptidase IV^[78] and $\text{Ca}^{2+}/\text{Mg}^{2+}$ -ATPase^[79]. Altered localization of Mrp2 and Bsep may represent aggravation of the secretory dysfunction caused by the parallel decrease in the hepatocellular content of the carriers that also occurs in this disease^[80,81], or even to be a causal factor of this reduction^[77,82-85]. Indeed, Paulusma *et al*^[77] have also found that, in contrast to that which is observed for Mrp2 protein content, mRNA levels are preserved after BDL, which suggests post-transcriptional downregulation of Mrp2 expression. They have postulated that endocytic internalization may represent the primary step toward enhanced breakdown of the endocytosed carriers. If maintained with time in chronic cholestatic conditions, this may cause redirection of the protein to the lysosomal compartment, followed by degradation.

The events leading to endocytic internalization of Mrp2 and Bsep in BDL rats remain uncertain. It is likely that accumulation of bile salts or other endogenous, potentially toxic compounds in the liver represents a causal factor. Bile salts are able to trigger oxidative stress^[86,87], which in turn may explain the release of pro-inflammatory cytokines in BDL rats^[88]. Both events have been involved in canalicular transporter internalization, as described below. We have found that the alteration in the normal pattern of localization of Mrp2, and that of the tight-junctional protein occludin, does not occur until 4 h after BDL in rats^[78], in contrast

to the immediate response observed in drug-induced cholestasis (see next section). This suggests that BDL alterations are secondary to intracellular accumulation of deleterious endogenous compounds.

Drug-induced cholestasis: Administration to laboratory animals of drugs known to induce functional, hepatocellular cholestasis, or administration of endogenous compounds thought to be the etiological factors of human cholestatic liver diseases, has been used as an experimental tool to study the mechanisms of the disease. Administration of the cholestatic, naturally-occurring estrogen estradiol-17 β -d-glucuronide (E2-17G)^[89,90], the cholestatic monohydroxylated bile salt tauroolithocholate (TLC)^[91,92] and the cholestatic immunosuppressor drug cyclosporine A^[93] all induce cholestasis in a short-term fashion, accompanied by endocytic internalization of Mrp2 and Bsep.

We have characterized in detail the mechanisms of transporter internalization in E2-17G-induced cholestasis, an experimental model that reproduces in part pregnancy-induced cholestasis. After a single, i.v. administration of this compound, bile flow decreases in a dose-dependent fashion with a nadir at 20 min, and spontaneously recovers to normality by 2 h post-injection^[94]. The cholestatic phase is associated with endocytic internalization of Mrp2 and Bsep, whereas the recovery phase occurs in parallel with the spontaneous re-insertion of subapical vesicles into the canalicular membrane^[89,90]. While the internalization process is microtubule-independent, re-insertion is microtubule-dependent, and stimulated by cAMP^[95]. We also found that repeated administration of E2-17G to rats leads to both a deeper internalization of Mrp2 and an abnormal localization of a small fraction to the lateral membrane^[78]. The latter phenomenon likely reflects loss of the fence between apical and basolateral domains caused by the simultaneous alteration of the tight-junctional structures^[95,96]. Unlike Mrp2 and Bsep, AQP8 has a preserved localization in E2-17G-induced cholestasis, and, like Mrp2 and Bsep, this water channel has a dual (intracellular plus plasma membrane) localization^[97].

Lipopolysaccharide (LPS)-induced cholestasis: LPS is an endotoxin localized in the outer membrane of Gram-negative bacteria. The toxin induces cholestasis mainly by the release of pro-inflammatory cytokines, such as tumor necrosis factor- α and interleukin-1 by monocytes/macrophages and, in the liver, Kupffer cells^[98]. Administration of LPS to laboratory animals represents, therefore, a good experimental model of inflammatory cholestatic diseases, not only of those caused by endotoxemia, but also those related to hepatitis caused by alcohol, autoimmune disease or drug intake.

LPS administration leads to endocytic internalization of Mrp2 and Bsep, which relocates in intracellular vesicular structures^[99-101]. The time-dependency of the effect of LPS on Mrp2 internalization has been

characterized by Kubitz *et al*^[101]. These authors have found that, 3 h after LPS treatment, Mrp2 is found in intracellular vesicles in the vicinity of the canalicular membrane, and that these vesicles are deeply internalized after 6-12 h treatment. Endocytic internalization of ABC canalicular transporters seems to be specific, as localization of the canalicular enzyme dipeptidyl peptidase IV is not affected by the treatment. Mrp2 internalization is reversed by perfusing the liver with a hypo-osmotic buffer, a maneuver known to stimulate exocytic insertion of canalicular transporters under normal conditions^[99,102]. However, this rescue of transporters occurs within 3 h of LPS administration, but not later on. It is possible that reversibility of the endocytic process depends on the degree of internalization of Mrp2, and that sustained internalization leads to delivery of the protein to the lysosomal compartment, followed by degradation. LPS effects can be prevented by administration of glucocorticoids^[101] or by heat stress^[103,104], two maneuvers that cause a decrease in synthesis and/or release of pro-inflammatory cytokines.

Oxidative-stress-induced cholestasis: Oxidative stress is a common feature in most liver diseases^[105]. Radical oxygen species induce biliary secretory failure and cholestasis, even at low, pre-necrotic levels^[106], and endocytic internalization of canalicular transporters may play a key role. We have shown that Bsep undergoes endocytic internalization into intracellular vesicles in isolated rat hepatocyte couplets when exposed to low levels of the pro-oxidizing compound tert-butylhydroperoxide (tBOOH)^[107]. This is accompanied by a reduced capability to accumulate the fluorescent bile salt analogue CLF in their canalicular vacuoles. A similar phenomenon has been described for Mrp2 after exposure of isolated perfused rat livers to the pro-oxidant agents tBOOH^[108], chloro-dinitrobenzene^[108] and ethacrynic acid^[109,110], or after hepatic ischemia-reperfusion^[111].

Endocytic internalization of transporters in human cholestatic liver disease

Changes in canalicular export pumps have been shown to occur in many human cholestatic liver diseases. Unlike the situation in rodents, downregulation of the expression of these transporters in human cholestatic disease is mostly post-transcriptional in nature, therefore, internalization of these transporters followed by degradation may represent a crucial mechanism to explain the disease in humans.

Internalization of canalicular export pumps has been observed in virtually all kinds of human cholestasis, including: (1) obstructive extrahepatic cholestasis^[112,113]; (2) inflammatory cholestasis associated with autoimmune hepatitis^[113]; (3) mixed (obstructive plus inflammatory) cholestatic disease, such as primary biliary cirrhosis^[114] and primary sclerosing cholangitis^[113]; and (4) acute cholestasis induced by drugs, such as that triggered by

antibiotics, tiopronin, chlorpromazine and non-steroidal anti-inflammatory drugs^[113,115]. Patients with obstructive cholestasis that are subjected to percutaneous transhepatic biliary drainage show different degrees of transporter dyslocalization, depending on the efficacy of the biliary drainage^[112,113], which points to a central role for retained endogenous compounds in this pathomechanism.

Mechanisms of endocytic internalization in cholestasis: role of signaling pathways

The mechanisms by which endocytosis of canalicular transporters occurs in cholestasis remains poorly understood. At least in part, this may be because they are multifactorial.

Alterations of actin-cytoskeletal integrity by administration of the F-actin poison phalloidin^[116], or secondary to the administration of pro-oxidant compounds, such as *t*-BOOH^[107] or the hydrophobic bile salts taurochenodeoxycholate^[117], triggers canalicular transporter endocytosis. This may be related to the fact that actin cytoskeleton is involved in transcytosis processes by operating as a bridge between microtubules and the apical membrane itself, in a coordinated action of the microtubule- and the F-actin-based motor proteins, kinesin and myosin, respectively^[118]. However, internalization of canalicular transporters also occurs with preserved actin organization, e.g. in E2-17G-^[89,90] or TLC-^[92] induced cholestasis. In these cases, components of the microfilament network other than actin, but associated with it, may be independently affected. Actin can interact with, and possibly regulate, transmembrane proteins *via* binding to plasma membrane actin cross-linking proteins, such as the ezrin-radixin-moesin (ERM) family of proteins, or by binding to interacting-partner proteins, such as PDZK1 and HAX-1. These cytoskeleton-associated proteins are required for the biosynthetic targeting of transmembrane proteins from the *trans*-Golgi network to the proper membrane domain, and for their further cell-surface retention^[119-121]. Mice that lack radixin, the main ERM protein in liver, develop conjugated hyperbilirubinemia associated with retrieval of Mrp2^[122]. Furthermore, downregulation of radixin using interfering RNA technology in collagen-sandwich-cultured rat hepatocytes disturbed the normal development of canalicular structures, and dissociated canalicular export pumps from their normal location at the apical membrane. Inside the cell, the transporters are found to be largely associated with Rab11-containing endosomes^[123]. Furthermore, a disturbed co-localization of MRP2/Mrp2 and radixin associated with endocytic internalization of the carrier is apparent in obstructive and estrogen-induced cholestasis in rats^[124], and in several cholestatic liver diseases in humans, including primary biliary cirrhosis stage III, drug-induced liver injury, obstructive jaundice, primary sclerosing cholangitis and autoimmune hepatitis^[113,114]. On the contrary, alteration in cholestasis of the localization/function of interacting-partner proteins, such as PDZK1 (for Mrp2) and HAX-1 (for Bsep, Mdr2 Mrp1) remains

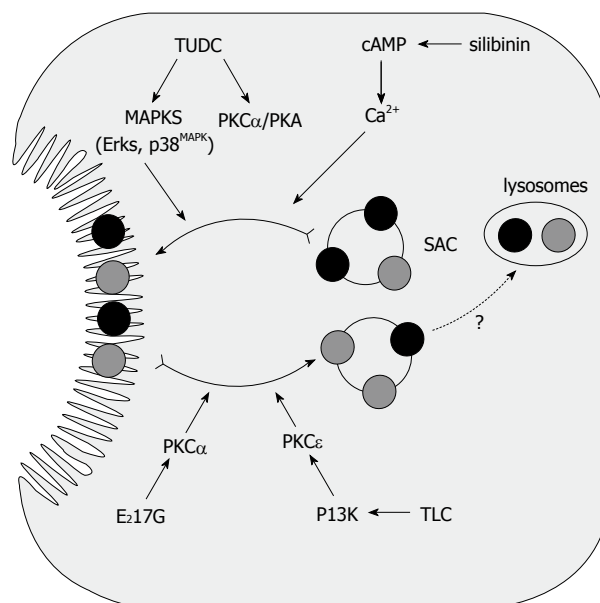


Figure 6 Endocytic internalization of canalicular transporters in E2-17G and in TLC-induced cholestasis. Protection from these cholestatic agents by the anticholestatic agents cAMP and TUDC is also shown. E2-17G and TLC induce endocytic internalization of canalicular transporters into the subapical compartment (SAC); this may lead to delivery to the lysosomal compartment, followed by degradation. E2-17G-induced activation of PKC α and TLC-induced, phosphatidylinositol 3-kinase (PI3K)-dependent activation of PKC ϵ have been proposed to mediate this retrieval. Elevation of intracellular cAMP levels induced by administration of the permeant cAMP analogue DBcAMP, or by the phosphodiesterase inhibitor silibinin, prevents internalization, and accelerates re-insertion, *via* cytosolic Ca²⁺ elevations. On the other hand, TUDC prevents transporter endocytosis probably *via* co-stimulation of PKC α - and PKA-dependent pathways.

to be confirmed. This possibility however exists, since retention of Mrp2^[122] and Oatp1a1^[125] in the apical and the basolateral membranes, respectively, requires interaction with the PDZ-domain protein, PDZ1. In addition, there is evidence that HAX-1 participates in clathrin-mediated Bsep endocytosis from the canalicular plasma membrane^[126].

Accumulating evidence indicates that changes in canalicular transporter localization that occur in cholestasis also depend on activation of critical intracellular signaling pathways (Figure 6). Representative examples are cPKC (mainly, PKC α in hepatocytes). Selective activation of cPKC induces endocytic internalization of Bsep from the canalicular membrane and cholestasis in the isolated perfused rat liver^[58]. Coincidentally, pan-specific activation of PKC also induces redistribution of MRP2 from the canalicular to the basolateral membrane in HepG2 cells^[127]. A critical participation of cPKC in the endocytic internalization of Bsep and the associated bile-salt secretory failure has recently been demonstrated by our group in E2-17G-induced cholestasis in rats^[128]. A similar role for cPKC has also been reported in cholestasis associated with *t*-BOOH-induced oxidative stress^[107] (Figure 7). However, under oxidative stress, the type of canalicular protein that is internalized and the signaling molecule involved seem to depend on the magnitude of the oxidative challenge. Low concentrations of the oxidizing

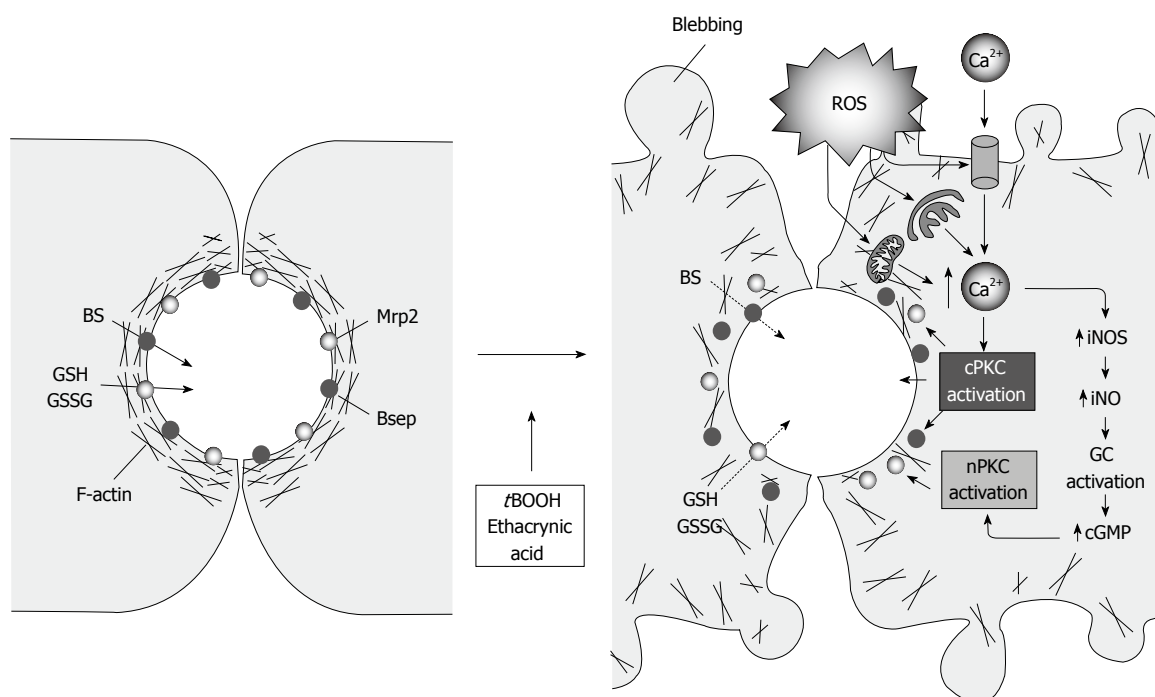


Figure 7 Endocytic internalization of canalicular transporters under oxidative stress. In normal cells, the pericanalicular arrangement of F-actin allows for the appropriate insertion of the canalicular transporters in their membrane domain. Reactive oxygen species produced by the administration of oxidizing compounds, such as tBOOH or ethacrynic acid, induces mobilization of Ca^{2+} across the plasma membrane and membranes of the calciosome (smooth ER and mitochondria), and the subsequent activation of cPKC. cPKC activation induces blebbing and redistribution of F-actin from the pericanalicular region to the cell body. This rearrangement, in turn, leads to canalicular transporter internalization. Moderate Ca^{2+} elevations may also activate iNOS, which induces NO-mediated guanylate cyclase activation and further cGMP-mediated activation of nPKC, which may internalize selectively Mrp2.

compound, ethacrynic acid, does not translocate cPKC, but novel PKC isoforms (nPKC). Under these conditions, the compound internalizes selectively Mrp2 without affecting Bsep, by a mechanism that probably involves Ca^{2+} -dependent activation of inducible nitric oxide (NO) synthase (iNOS), followed by NO-mediated cGMP increase, and further cGMP-activation of nPKC^[110]. However, higher doses of ethacrynic acid, sufficient to activate cPKC isoforms, induce internalization of Bsep and Mrp2^[110].

The nPKC isoform PKC ϵ is also activated in TLC-induced cholestasis, and has been suggested to be involved in the TLC cholestatic effect^[129]. This phenomenon occurs in a PI3K-dependent manner, which is consistent with the finding that PI3K products are potent activators of PKC ϵ ^[130] (Figure 6). Since PI3K has been also shown to have pro-insertion properties (see above), this may be regarded as paradoxical. However, pro-exocytic and pro-endocytic effects of PI3K have been inferred by using pan-specific inhibitors of PI3K, and different isoforms of this kinase may have accounted for by these different effects.

Anticholestatic therapeutic approaches based upon modulation of dynamic carrier localization

As illustrated above for E217G-induced cholestasis, internalization of hepatocellular transporters in cholestasis is spontaneously reversed if the cholestatic insult is transient. This spontaneous recovery occurs by a microtubule-dependent re-targeting of the endocytosed

transporters to the canalicular membrane^[95]. Some experimental therapeutic approaches have been designed to prevent transporter internalization and/or to accelerate this re-insertion, so as to avoid irreversible consequences of sustained internalization (Figure 6). The therapeutic agents studied include the following.

cAMP: This second messenger partially prevents the impairment of bile flow and internalization of ABC transporters in experimental cholestasis, consistent with its capability to stimulate vesicle-mediated targeting of canalicular transporters^[54,55,60]. The drop in bile flow and transport activity of Bsep^[90] and Mrp2^[89] in the acute phase of E217G-induced cholestasis can be partially prevented by cAMP. More significantly, cAMP shortens spontaneous recovery to normality of bile flow, Mrp2 function and Mrp2 localization^[89]. A similar acceleration of the re-insertion of endocytosed transporters has been described by our group for Bsep in TLC-induced cholestasis^[92]. In isolated rat hepatocyte couplets, a preventive effect of cAMP has been observed in E217G-^[90,131] and TLC^[92,131]-induced Bsep mislocalization. In this case, however, prevention by cAMP is complete. This protective effect is significantly blocked by the Ca^{2+} chelator, BAPTA/AM, but not by the PKA inhibitor, KT5720, which suggests involvement of Ca^{2+} -dependent signaling pathways. A similar anticholestatic mechanism in terms of the signaling modulators involved is afforded by silibinin, the active component of the hepatoprotector silymarin^[131]. This most likely

results from the capability of silibinin to inhibit cAMP phosphodiesterase, thus increasing endogenous cAMP intracellular levels^[131].

TUDC: This taurine-conjugate bile salt stimulates exocytic insertion of canalicular export pumps as part of its choleretic effect^[63], and counteracts endocytic internalization of Bsep^[134] and Mrp2^[91] in TLC-induced cholestasis (Figure 6). The Ca²⁺-sensitive, PKC isoform, PKC α , has been proposed to mediate its anticholestatic effect^[91], *via* a cooperative PKC α /PKA-dependent mechanism^[133]. This is in apparent contradiction with more recent findings that PKC α is cholestatic rather than hepatoprotective^[58]. However, the biological response evoked by the interplay between different protein kinases (PKC α /PKA) may be different from that evoked by just one of them (PKC α). Furthermore, TUDC activates Erk^[64] and p38^{MAPK}^[63], and the cholestatic effect of PKC α may be overridden by the choleretic effects of these signal transduction pathways.

4-Phenylbutyrate (4-PBA): This compound has been shown to restore the reduced cell surface expression of cystic fibrosis transmembrane conductance regulator in cystic fibrosis patients, who have mutated forms of the protein, which suggests improved targeting of the transporter to its membrane domain. When the 4PBA-proinserting property was tested for Bsep in normal rats, it was observed that canalicular expression and bile-salt transport function were improved by this compound^[134]. A possible mechanism that 4PBA treatment increases the cell-surface-resident Bsep is the interruption of the internalization process from the cell surface to the intracellular compartment, or promotion of recycling from the intracellular compartment back to the cell surface^[134]. Stabilization of Bsep in the membrane by 4PBA has also been confirmed in MDCK cells for wild-type Bsep and Bsep with E297G and D482G mutations, which occurs in progressive familial intrahepatic cholestasis type 2 (PFIC2). Since trafficking of these Bsep-mutated proteins is impaired in PFIC2^[135], this agent may be a potential candidate to halt the progression of this genetic disease. Its efficacy in acquired cholestatic diseases remains to be ascertained.

FUTURE DIRECTIONS

The overwhelming progress in molecular biology techniques and the availability of *in vitro*, polarized cell models for the study of hepatobiliary function has greatly facilitated the characterization at a molecular level of the mechanism involved in the sorting of hepatobiliary transport systems from their sites of synthesis, and their recycling from/to endosomal compartments available on demand. However, the increasing number of new cytoskeletal, motor and signaling proteins that are being discovered as a result of these technological developments makes the characterization of their role in transporter trafficking an endless challenge.

Advances in the molecular field have promoted a parallel progress in the understanding of the consequences that the alterations in the mechanisms of trafficking have in liver disease. It is becoming increasingly evident that impairment in the dynamic localization of hepatocellular transporters is a common feature in hepatocellular cholestasis. However, the characterization of the molecular mechanisms that underlie this alteration is in its infancy. Many crucial questions remain to be answered, for example: (1) which are the signaling mediators that trigger endocytosis of canalicular transporters in each kind of cholestasis; (2) which are the molecular targets of these cholestatic mediators that ultimately govern carrier internalization?; and (3) can changes in localization of these transporters be not only prevented but, what is more important from the therapeutic point of view, reversed by factors that counteract these dysfunctions? Satisfactory answers to these questions would allow the design of new therapeutic strategies in cholestatic liver diseases to assure proper localization of transporters in an attempt to prevent their accelerated degradation. We hope that progress in experimental therapeutics based on this current information encourages clinical researchers to apply this knowledge to envisage better, innovative therapeutic alternatives for the treatment of human cholestatic liver disease.

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LIVER CANCER

Inhibitory effect of interferon- α -2b on expression of cyclooxygenase-2 and vascular endothelial growth factor in human hepatocellular carcinoma inoculated in nude mice

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Abstract

AIM: To evaluate the effects of interferon- α -2b (IFN- α -2b) on expression of cyclooxygenase-2 (COX-2) and vascular endothelial growth factor (VEGF) in human hepatocellular carcinoma (HCC) inoculated in nude mice and to study the underlying mechanism of IFN- α -2b against HCC growth.

METHODS: Thirty-two nude mice bearing human HCC were randomly divided into four groups ($n = 8$). On the 10th day after implantation of HCC cells, the mice in test groups (groups A, B and C) received IFN- α -2b at a serial dose (10000 IU for group A, 20000 IU for group B, 40000 IU for group C sc daily) for 35 d. The mice in control group received normal saline (NS). The growth conditions of transplanted tumors were observed. Both genes and proteins of COX-2 and VEGF were detected by RT-PCR and Western blot. Apoptosis of tumor cells in nude mice was detected by TUNEL assay after treatment with IFN- α -2b.

RESULTS: Tumors were significantly smaller and had a lower weight in the IFN- α -2b treatment groups than those in the control group ($P < 0.01$), and the tumor growth inhibition rate in groups A, B and C was 27.78%, 65.22% and 49.64%, respectively. The expression levels of both genes and proteins of COX-2 and VEGF were much lower in the IFN- α -2b treatment groups than in the control group ($P < 0.01$). The

apoptosis index (AI) of tumor cells in the IFN- α -2b treatment groups was markedly higher than that in the control group ($P < 0.01$). Group B had a higher inhibition rate of tumor growth, a lower expression level of COX-2 and VEGF and a higher AI than groups A and C ($P < 0.05$), but there was no significant difference between groups A and C.

CONCLUSION: The inhibitory effects of IFN- α -2b on implanted tumor growth and apoptosis may be associated with the down-regulation of COX-2 and VEGF expression. There is a dose-effect relationship. The medium dose of IFN- α -2b for inhibiting tumor growth is 20000 IU/d.

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Key words: Hepatocellular carcinoma; Interferon- α -2b; Cyclooxygenase-2; Vascular endothelial growth factor; Apoptosis

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INTRODUCTION

In recent years, studies have proved the inhibitory effects of interferon- α -2b (IFN- α -2b) on many kinds of tumors^[1-3], but the effects of IFN- α -2b on expression of cyclooxygenase-2 (COX-2) and vascular endothelial growth factor (VEGF) in hepatocellular carcinoma (HCC) have not been extensively studied. This study investigated the effects of IFN- α -2b on COX-2 and VEGF expression in human HCC implanted in nude mice and the underlying mechanism of its inhibitory effect on the growth of HCC.

MATERIALS AND METHODS

Reagents

HCC cell line HepG2 was stored in our laboratory. Rabbit anti-human COX-2 polyclonal antibody (Santa Cruz, US) was purchased from Beijing Zhongshan Biotechnology Company, Ltd. Mouse anti-human VEGF polyclonal antibody (Neomarkers, USA), TRIzol and RT-PCR kits were from Jinmei Biotech Company, Ltd. Primers of RT-PCR were synthesized by Alpha Biotechnology Company (Wuhan, China). *In situ* apoptosis detection kits (Boehringer Mannheim, Germany) and ECL chemiluminescence detection kits (Pierce, US) were from Beijing Zhongshan Biotechnology Company, Ltd. IFN- α -2b injection (trade name: Interlong) was from Shenzhen Neptunus Interlong Bio-tech Holdings Company, Ltd (China).

Animal model

A transplantation tumor model of human HCC in nude mice was established. In brief, thirty-two 4-6 wk old female BALB/c nu/nu mice, weighing 18-20 g, bred in SPF rooms, were obtained from the Experimental Animal Center of Tongji Medical University, Huazhong University of Science and Technology (Wuhan, China). The concentration of single cell suspension of HepG2 cells was adjusted to 5×10^5 /L at the exponential growth phase. The cell viability was kept above 95% assessed by Typan blue exclusion. Then, the cell suspension was inoculated subcutaneously in the back of nude mice (0.2 mL/each mouse). The standard of tumor formation was defined as its nodus diameter (up to 0.5 cm). The rate of tumor formation was 100% 10 d after inoculation. The mice meeting the standard of tumor formation were randomly divided into 4 groups ($n = 8$).

Drugs and groups

Each nude mouse meeting the standard of tumor formation in groups A, B and C received 10 000 IU IFN- α -2b, 20 000 IU and 40 000 IU of IFN- α -2b, respectively, once a day for 35 d. Each nude mouse in control group received only 0.1 mL normal saline (NS), once a day for 35 d. Then, the mice were killed. The maximum and minimum diameters of tumor were measured with a sliding caliper and the weight of tumor was recorded. A certain number of samples were stored at -70°C . The rest samples were fixed in 10% formalin, imbedded in paraffin, and cut into serial sections for assay of *in situ* apoptosis. Volume of tumor (V) = $ab^2/2$, inhibition ratio (%) = [(mean tumor weight of control group - mean tumor weight of test group) / mean tumor weight of control group] $\times 100\%$.

RT-PCR assay

Total RNA was extracted with the TRIzol through one-step method, and its concentration and purity were detected using a DNA/RNA detector (Pharmacia Company, British). cDNA was synthesized by a reverse transcription reaction of 4 μg of total RNA with oligo-

(dt)₁₅ primers and retroviridase at 45°C for 60 min, and stored at -20°C .

The sequences of primers used for semi-quantitative PCR are as follows: sense: 5'-CAAGTCCCTGAGCATCTACG-3' and anti-sense: 5'-CATTCCTACCACCAGCAACC-3' for COX-2; sense: 5'-TTGCTGCTCTACCTCCAC-3' and anti-sense: 5'-AATGCTTTCTCCGCTCTG-3' for VEGF; sense: 5'-CAGAGCAAGAGAGGCAGCCT-3' and anti-sense: 5'-GGATAGCACAGCCTGGATAG-3' for β -actin. PCR conditions were as follows: 30 cycles at 94°C for 1 min, at 54°C for 30 s, and at 72°C for 1 min for COX-2; 27 cycles at 94°C for 1 min, at 56°C for 30 s, and at 72°C for 1 min for VEGF; 30 cycles at 94°C for 1 min, at 59°C for 30 s, and at 72°C for 1 min for β -actin. The lengths of PCR products of COX-2, VEGF and β -actin were 490 bp, 417 bp and 250 bp, respectively. After separated by electrophoresis on 1.5% agarose gel, the bands of PCR products were scanned and a densitometric analysis was performed with a MUVB-20 gel analysis system (Ultralum Company, USA). The relative expression levels of COX-2 mRNA and VEGF mRNA were represented as absorbance ratios (COX-2/ β -actin and VEGF/ β -actin).

Western blot assay

The protein was extracted from tumor tissue through three-detergent methods. An equal quantity of protein was separated from each sample by electrophoresis on 10% SDS-PAGE gel, and then transferred to a nitrocellulose filter (NC filter) with a semidry transfer cell. The NC filter containing protein samples was incubated in a blocking buffer containing 5% nonfat dry milk for 2 h, then with the first antibody at a dilution of 1:250 in a blocking buffer overnight at 4°C . After washed with 0.1% TBS, the membrane was incubated at room temperature for 1 h with the second antibodies at a dilution of 1:5000 in a blocking buffer, then washed three times with 0.1% TBS, visualized, photographed and fixed through ECL according to its manufacturer's instructions. The absorbance value of protein bands was assessed with an image analysis system.

TUNEL assay

Paraffin-embedded tumor samples were cut into serial 4-5 μm thick serial sections, heated overnight at 58°C , and routinely deparaffinized and rehydrated. After digested by protease K for 15 min, the sections were incubated with TUNEL at 37°C for 1 h. After digoxin-marked alkaline phosphatase antibody was added, the sections were colored with BCIP/NBT, dehydrated with alcoholic, air dried, and mounted. Positive cells were defined as hyacinthine particles distributing in nuclei. Five high power fields were randomly selected from each section. Results were expressed as mean values. Apoptosis index (AI) = [the number of apoptosis cells / (the number of apoptosis cells + the number of unapoptosis cells)] $\times 100\%$.

Table 1 Inhibitory effect of IFN α -2b on transplanted HCC in nude mice (mean \pm SD, $n = 8$)

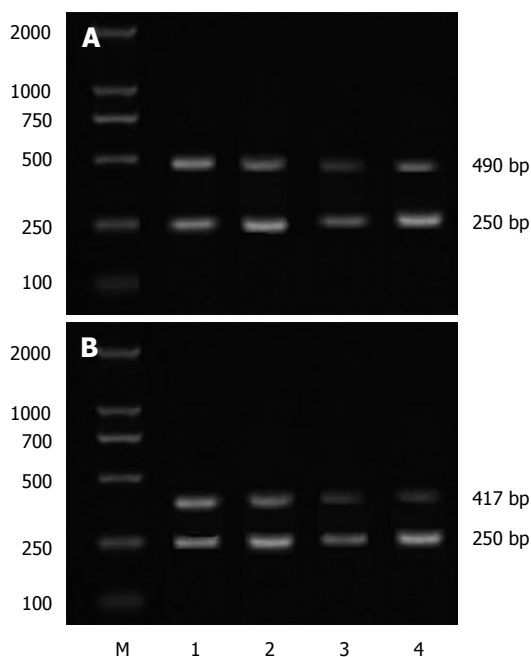
Groups	Dose (IU/d)	Tumor vol (mm ³)	Tumor Wt (mg)	Inhibition ratio (%)
Control	0	3727.11 \pm 745.53	1362.32 \pm 272.12	
Group A	10000	2082.18 \pm 416.43 ^a	983.85 \pm 193.76 ^a	27.78
Group B	20000	1521.43 \pm 313.89 ^{a,d}	473.76 \pm 92.57 ^{a,d}	65.22
Group C	40000	1806.35 \pm 363.71 ^a	686.06 \pm 138.21 ^a	49.64

^a $P < 0.01$ vs control group; ^d $P < 0.05$ vs groups A and C.

Table 2 Expression level of COX-2 and VEGF mRNA and protein in transplanted HCC of nude mice after treatment with IFN α -2b (mean \pm SD, $n = 8$)

mRNA				Protein			
Groups	Dose (IU/d)	COX-2	VEGF	Groups	Dose (IU/d)	COX-2	VEGF
Control	0	0.59 \pm 0.11	0.61 \pm 0.15	Control	0	0.75 \pm 0.16	0.82 \pm 0.18
Group A	10000	0.44 \pm 0.09 ^a	0.52 \pm 0.12 ^a	Group A	10000	0.52 \pm 0.09 ^a	0.61 \pm 0.13 ^a
Group B	20000	0.12 \pm 0.02 ^{a,d}	0.17 \pm 0.03 ^{a,d}	Group B	20000	0.23 \pm 0.04 ^{a,d}	0.31 \pm 0.05 ^{a,d}
Group C	40000	0.26 \pm 0.06 ^a	0.31 \pm 0.07 ^a	Group C	40000	0.38 \pm 0.07 ^a	0.43 \pm 0.08 ^a

^a $P < 0.01$ vs control group; ^d $P < 0.05$ vs groups A and C.

**Figure 1** RT-PCR showing expression levels of COX-2 mRNA (A) and VEGF mRNA (B). M: Marker.

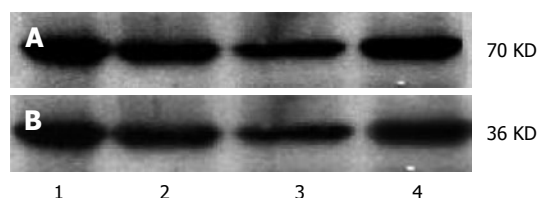
Statistical analysis

Data were presented as mean \pm SD. SPSS 11.5 was employed to analyze the data. The total difference among groups was analyzed by ANOVA. Q -test was used for the comparison between two groups. $P < 0.05$ was considered statistically significant.

RESULTS

Inhibitory effect of IFN- α -2b on transplanted tumors of nude mice

The tumor growth in three IFN- α -2b treatment groups was inhibited at different degrees. The inhibition rate of tumors in groups A, B and C was 27.78%, 65.22% and

**Figure 2** RT-PCR (A) and Western blot (B) showing expression levels of COX-2 and VEGF protein. 1: Control group; 2-4: Groups A, B, and C.

49.64%, respectively. The weight and volume of tumors were lower in three IFN- α -2b treatment groups than in control group ($P < 0.01$). The inhibitory effect was the greatest in group B, and significantly different from that in groups A and C ($P < 0.01$), but the difference was not statistically significant between groups A and C (Table 1).

Expression of COX-2 and VEGF mRNA in transplanted tumors of nude mice

RT-PCR showed that the expression levels of COX-2 and VEGF mRNA were lower in three IFN- α -2b treatment groups than in control group ($P < 0.01$). Meanwhile, their expression levels were significantly lower in group B than in groups A and C ($P < 0.05$), but the difference was not statistically significant between groups A and C (Table 2, Figure 1).

Expression of COX-2 and VEGF protein in transplanted tumors of nude mice

Western blot assay showed that the expression levels of COX-2 and VEGF protein were lower in three IFN- α -2b treatment groups than in control group ($P < 0.01$). Statistical analysis revealed similar results of COX-2 and VEGF mRNA in three IFN- α -2b treatment groups (Table 2, Figure 2).

Apoptosis of transplanted tumors of nude mice

TUNEL assay showed that the AI of tumor cells in the

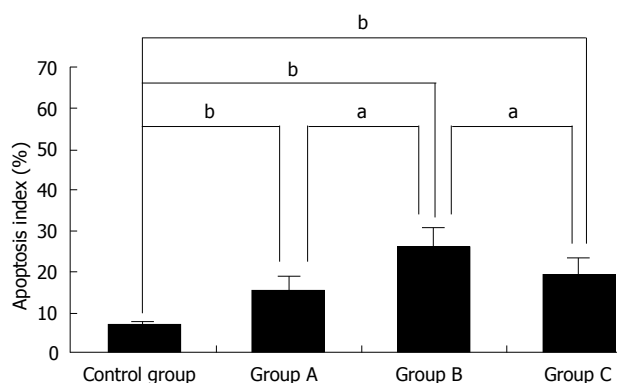


Figure 3 Changes in AI of transplanted HCC cells of nude mice. ^a $P < 0.05$, ^b $P < 0.01$ vs groups A and C.

4 groups was $6.39\% \pm 1.33\%$, $15.39\% \pm 3.09\%$, $25.53\% \pm 4.98\%$ and $19.28\% \pm 3.82\%$, respectively, and was significantly higher in three IFN- α -2b treatment groups than in control group ($P < 0.01$). However, the AI in group B was higher than that in groups A and C ($P < 0.05$), but the difference was not statistically significant between groups A and C (Figures 3-4).

DISCUSSION

HCC is one of the malignant tumors that can lead patients to death^[1], but the molecular mechanism of hepatocarcinogenesis has not been well understood. COX can be divided into two subtypes: COX-1 and COX-2. COX-2 is a rate-limiting enzyme of prostaglandin (PG) biosynthesis, encodes for 603 or 604 amino acids including 17 signal peptides of amino acid residues, contains 4 oligoses and 2 bands (molecular weight = 72 KDa and 74 KDa, respectively) in SDS-PAGE. Oshima *et al*^[2] showed that the expression of COX-2 is up-regulated in colon tumors and its activity plays an important role in the process of tumorigenesis. Besides, the expression of COX-2 is also higher in liver, breast, tongue, prostatic, cutaneous and mouth dermoid cancers, *etc*^[3-5].

It has been recently proved that COX-2 is also expressed in HCC at different degrees and HCC patients with a higher expression level of COX-2 have a higher recurrence rate, early metastasis and worse prognosis^[6,7], suggesting that over-expression of COX-2 may be related with a worse prognosis of HCC. The five-year survival rate of HCC patients after operation is only 25%-39%. Some studies indicate that over-expression of COX-2 may promote oncogenesis by regulating the expression of some genes related to cell proliferation and apoptosis^[6,7]. The underlying mechanism of COX-2 against oncogenesis may be through influencing many kinds of prostaglandin and thromboxan to bind to their corresponding receptors.

Angiogenesis plays an important role in tumorigenesis and tumor development. If no angiogenesis occurs, the volume of tumor can hardly be larger than 1-2 mm³. The degree of angiogenesis depends on the rate of angiogenesis and anti-angiogenesis factors in tumor

cells and adjacent host cells. The angiogenesis factors mainly include bFGF, VEGF, IL-8, MMP-2, MMP-9, *etc*. Among them, VEGF is most important, and plays a leading role in the growth, generation, recurrence and metastasis of tumors^[8-11]. As a dipolymer, glucoprotein has a molecular weight of 34-46 kDa. VEGF exerts its biological effect by binding specifically to Flt1 and KDR/Flk1 existing on the surface of vascular endothelial cells which belong to RTKIII receptors. A recent study showed that angiogenesis and VEGF play an important role in the recurrence and metastasis of HCC^[12]. It was reported that the expression of VEGF is up-regulated in HCC and positively correlated with the recurrence and metastasis of HCC after operation^[13].

Interferon (IFN) regulates the activity of cytokines which control cell function and replication, and inhibits the activity of tumor cells in many organs and tissues^[14-16]. It has been shown that IFN- α decreases the sensitivity of vascular endothelial cells to VEGF and bFGF, as well as the vascularization of tumors^[17].

The results of this study indicate that IFN- α -2b could significantly inhibit the growth of HCC in nude mice. The volume and weight of transplanted tumors decreased evidently in IFN- α -2b treatment groups. The expression levels of COX-2 and VEGF mRNA and protein as well as the AI increased significantly in IFN- α -2b treatment groups, indicating that the expression levels of COX-2 and VEGF are closely related with the growth of tumor and apoptosis of tumor cells. IFN- α -2b can down-regulate the expression levels of COX-2 and VEGF, but its mechanism remains unknown. Singer *et al*^[18] reported that IFN- α exerts its effects through the NF- κ B signal transduction pathway, and inhibitors of NF- κ B can decrease the expression level of COX-2. A study about the effect of IFN- α on a signal transduction pathway showed that IFN- α could reduce the activity of NF- κ B^[19], suggesting that IFN- α -2b can decrease the activity of the NF- κ B signal transduction pathway, inhibit the expression of COX-2 and the growth of HCC, and induce apoptosis. It has been shown that IFN- α can down-regulate the expression levels of angiogenesis factors, such as bFGF, VEGF, MMP-9 and IL-8, in different tumors of humans, then inhibit angiogenesis and the growth of tumors^[20]. Tsujii *et al*^[21] investigated the correlation between COX-2 and angiogenesis of colon carcinoma and found that colon carcinoma cells could secrete angiogenesis factors, such as VEGF, bFGF, TGF- β 1 and PDGF, at a high concentration. Inhibitors of COX-2 can significantly depress the expression of angiogenesis factors, indicating that COX-2 can promote the growth of tumors, which is closely related with its effect on promoting angiogenesis. We hold that IFN- α -2b can inhibit HCC growth by inducing apoptosis of tumor cells through down-regulation of COX-2 expression and by inhibiting angiogenesis of tumors through down-regulation of VEGF expression. COX-2 may influence the expression of VEGF. The signal transduction between COX-2 and VEGF still needs to be further studied.

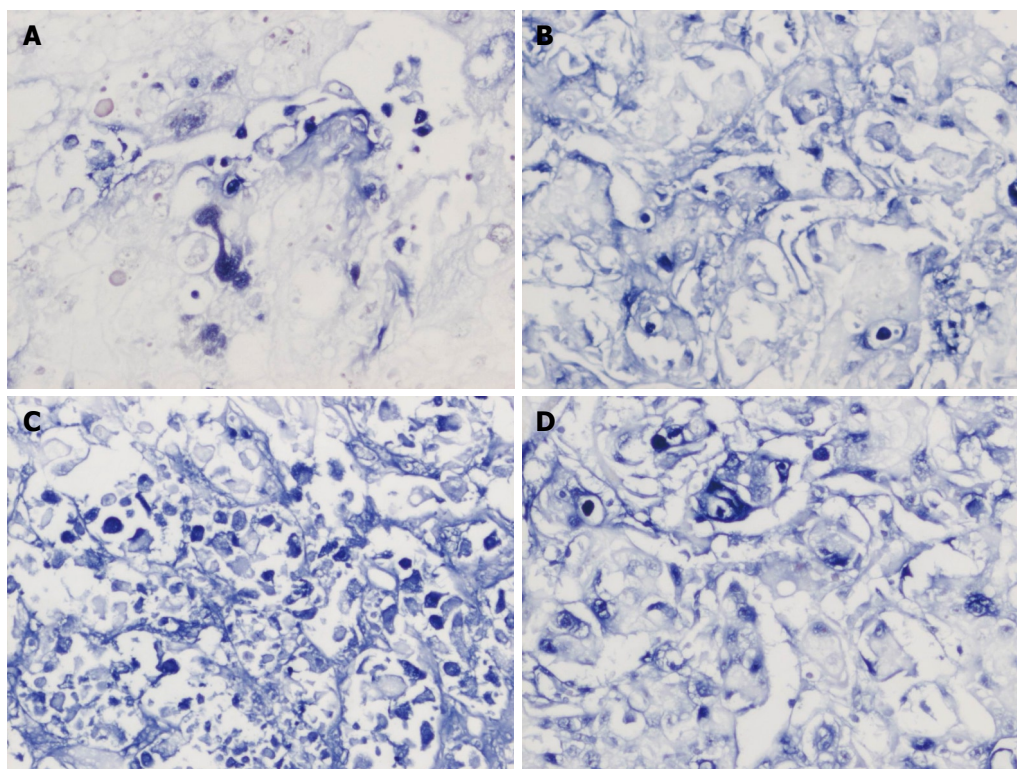


Figure 4 Apoptosis of transplanted HCC (TUNEL × 200). A: Control group; B: Group A; C: Group B; D: Group C.

In this study, the inhibitory effect of IFN- α -2b on HCC growth in nude mice showed a dose-effect relationship. The effect of IFN- α -2b was greater in group B than in groups A and C. However, there was no significant difference between groups A and C. Huang *et al*^[22] have reported a similar result in nude mice bearing carcinoma of prostate after treatment with pegylated IFN- α -2b (PEG-IFN- α -2b). At present, no common view is available on the dose of IFN α . Wang *et al*^[23] investigated the treatment of nude mice bearing orthotopically transplanted HCC with large doses of IFN- α (3×10^5 U/d and 6×10^5 U/d) and showed that IFN- α could inhibit metastasis and angiogenesis of HCC at a certain degree. Because the signal transduction of IFN is mediated by the JAK/STAT pathway, SOS protein could down-regulate the signals of IFN by blocking up the JAK/STAT pathway. Although studies are available on the activity of STAT3 inhibited by SOS3^[24-26], further study is needed to show the maximally tolerated dose of IFN- α and its correlation with the signal transduction of STAT1.

It has been shown that nonsteroidal anti-inflammatory drugs (NSAIDs) and INF- α have a synergistic effect on the HCC cell line HepG2. NSAIDs up-regulate the transcription of ISRE, increase the activity and inhibitory effect of IFN- α on tumors, thus providing a new treatment modality for HCC with IFN α ^[27].

COMMENTS

Background

In recent years, the inhibitory effect of interferon- α -2b (IFN- α -2b) on many tumors has been proved, but its effect on the expression of cyclooxygenase-2 (COX-2) and vascular endothelial growth factor (VEGF) in hepatocellular carcinoma (HCC) has rarely been reported.

Research frontiers

HCC is one of the malignant tumors that can lead to death of patients, but the molecular mechanism of hepatocarcinogenesis remains poorly understood.

Innovations and breakthroughs

Studies have proved the inhibitory effects of interferon- α -2b (IFN- α -2b) on many kinds of tumors. However, the effects of IFN- α -2b on expression of cyclooxygenase-2 (COX-2) and vascular endothelial growth factor (VEGF) in hepatocellular carcinoma (HCC) have not been extensively studied. This study investigated the effects of IFN- α -2b on COX-2 and VEGF expression in human HCC implanted in nude mice and the underlying mechanism of its inhibitory effect on the growth of HCC.

Applications

This study investigated the effects of IFN- α -2b on COX-2 and VEGF expression in human HCC implanted in nude mice and the underlying mechanism of its inhibitory effect on the growth of HCC. The results show that IFN- α -2b can be used as an effective agent in the treatment of HCC.

Peer review

The study is well designed. The results of this study are interesting and seem to be reliable.

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BASIC RESEARCH

Failure of P-selectin blockade alone to protect the liver from ischemia-reperfusion injury in the isolated blood-perfused rat liver

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injury in the isolated blood-perfused cold-*ex vivo* rat liver model.

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Key words: P-selectin; Ischemia-reperfusion; Antibody-blockade; Liver; Rat

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Wyllie S, Barshes NR, Gao FQ, Karpen SJ, Goss JA. Failure of P-selectin blockade alone to protect the liver from ischemia-reperfusion injury in the isolated blood-perfused rat liver. *World J Gastroenterol* 2008; 14(44): 6808-6816 Available from: URL: <http://www.wjgnet.com/1007-9327/14/6808.asp> DOI: <http://dx.doi.org/10.3748/wjg.14.6808>

Abstract

AIM: To determine if blockade of P-selectin in the isolated blood-perfused cold *ex vivo* rat liver model protects the liver from ischemia-reperfusion injury.

METHODS: The effect of P-selectin blockade was assessed by employing an isolated blood-perfused cold *ex vivo* rat liver with or without P-selectin antibody treatment before and after 6 h of cold storage in University of Wisconsin solution.

RESULTS: In our isolated blood-perfused rat liver model, pre-treatment with P-selectin antibody failed to protect the liver from ischemia-reperfusion injury, as judged by the elevated aspartate aminotransferase activity. In addition, P-selectin antibody treatment did not significantly reduced hepatic polymorphonuclear leukocyte accumulation after 120 min of perfusion. Histological evaluation of liver sections obtained at 120 min of perfusion showed significant oncotic necrosis in liver sections of both ischemic control and P-selectin antibody-treated groups. However, total bile production after 120 min of perfusion was significantly greater in P-selectin antibody-treated livers, compared to control livers. No significant difference in P-selectin and ICAM-1 mRNAs and proteins, GSH, GSSG, and nuclear NF- κ B was found between control and P-selectin antibody-treated livers.

CONCLUSION: In conclusion, we have shown that blockade of P-selectin alone failed to reduced polymorphonuclear leukocyte accumulation in the liver and protect hepatocytes from ischemia-reperfusion

INTRODUCTION

Ischemia-reperfusion (I/R)³ injury has been shown to play a major role in clinical and experimental hemorrhagic shock, organ resection, and transplantation^[1-5]. The inflammatory component of I/R injury is mediated by pro-inflammatory cytokines such as TNF- α and IL-1 β , and cellular adhesion molecules such as β 2-integrins, ICAM-1, VCAM-1, and members of the selectin family, P-, E-, and L-selectin^[6-8]. The sequence of events currently enjoying the most popularity as the mechanism responsible for I/R injury of the liver is: (1) KC are activated following I/R^[9]; (2) During early reperfusion (0-2 h), KC are further activated by complement and produce significant vascular oxidative stress^[10]; (3) KC also produce pro-inflammatory cytokines and chemokines, which is dependent on the activation of the redox-sensitive transcription factor NF- κ B^[11]. Activated hepatocytes and endothelial cells also produce reactive oxygen species (ROS) and contribute to the liver cytokine-chemokine milieu; (4) Cytokine mediated induction of adhesion molecules such as P- and E-selectins, ICAM-1, and VCAM-1 on the liver endothelium occur during reperfusion; (5)

PMNs accumulate in the liver as a result of P- and E-selectin-mediated rolling and margination on the liver endothelium, followed by ICAM-1-dependent firm adhesion. Although PMNs accumulate in the liver during early reperfusion, they do not contribute to liver injury until the latter phase (6-24 h) of I/R injury^[10,12,13]; and (6) PMNs transmigrate to the liver parenchyma *via* ICAM-1 and VCAM-1, bind to hepatocytes *via* ICAM-1/ β 2-integrins (CD11b/CD18), and engage in a sustained production of ROS to produce intracellular oxidative stress in hepatocytes and cell death^[14-17].

Following I/R of several organs or tissues, a general mechanism of selectin-dependent rolling of PMNs followed by firmer adhesion to endothelial cells by integrins and ICAM-1 is applicable to their vasculature (heart, lung, intestine, and cremaster muscle). Accordingly, numerous studies reported that anti-P-selectin therapy afforded protection to the liver from I/R injury^[18-21]. However, this general mechanism may not be applicable to the liver^[13,14]. Numerous reports suggest that P-selectin attenuates I/R injury of the liver by mediating the recruitment of PMNs^[18-20], while other reports minimize its role in liver I/R injury and its role in recruiting PMNs in the inflamed liver vasculature^[21-26]. Furthermore, hepatic PMNs accumulation, mediated by P-selectin expressed on endothelial cells of postsinusoidal venules, might not contribute significantly to liver injury, because there is no experimental evidence supporting extravasation of these neutrophils to the liver parenchyma^[23,26]. In addition, a recent report by Kubes *et al.* suggest that the protective effect observed in the liver with anti-P-selectin therapy may be mostly secondary to the anti-P-selectin therapy of accompanying intestinal I/R injury^[27].

If the above scenario is to hold, then blockade of P-selectin should prevent or attenuate I/R injury, at least during the latter phase of I/R injury in the warm *in vivo* liver model. Therefore, to investigate if P-selectin blockade alone protects the liver from I/R injury, we employed an antibody to P-selectin and a cold-*ex vivo* I/R rat liver model. The present study demonstrates that while anti-P-selectin treatment may increase total bile flow in livers subjected to I/R, it failed to protect hepatocytes in the isolated blood-perfused rat liver model.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats (250-350 g) were purchased Charles Rivers, Houston, TX). All animals used in this study received a nutritionally balanced rodent diet, water *ad libitum*, and were cared for according to NIH guidelines.

Isolated-Perfused-Rat-Liver (IPRL) model

In brief, animals were anaesthetized with Nembutal (50-60 mg/kg *bd.* wt., *ip*, Sigma-Aldrich, St. Louis, MO), and under aseptic conditions, a laparotomy performed

to access the liver for mobilization. Livers were carefully isolated from male Sprague-Dawley rats under Nembutal anesthesia after cannulation of the portal vein, common bile duct, and suprahepatic vena cava, while constantly perfused with oxygenated Krebs-Hensleit buffer (pH 7.4) *via* the portal vein^[28]. Immediately after isolation, control and treated livers were flushed with 10 mL of pristine UW solution, and stored at 4°C for 6 h. Livers in the treated group received an additional flush of 1 mL of UW solution containing 420 μ g of P-selectin Ab (CD62P, Cat.#553716, PharMingen, San Diego, CA) *via* the portal vein before cold-*ex vivo* ischemia (storage) and immediately before perfusion. This antibody has been shown to inhibit the binding of neutrophils to rat P-selectin in both *in vitro* and *in vivo* studies. Control livers were also flushed with 1 mL of pristine UW solution immediately before perfusion. At the end of cold storage, livers were perfused with syngenic rat blood (diluted with Krebs-Hensleit buffer (pH 7.4) to a hematocrit of 12%, total volume 100 mL) in a re-circulating perfusion system using a fully-jacketed isolated-perfusion-rat-liver apparatus (RGT #130003, Radnoti Glass Technology, Inc., Monrovia, CA) for 120 min, as previously described^[10]. Prior to perfusion, the perfusion apparatus was primed with blood perfusate at 37°C. Oxygenation was done with a membrane-oxygenating chamber (PO₂ held > 250 mm Hg) monitored with inline-digital pressure transducer. Portal vein perfusate flow was continually adjusted to maintain portal pressures between 18 and 23 mm Hg, and monitored with inline-digital pressure transducer. Temperature, pH, and oxygen level were maintained throughout each experiment. Liver sections (snap-frozen in liquid nitrogen), blood perfusate, and bile (collected in pre-weighed eppendorf tubes) were collected every 30 min during perfusion. At the end of each experiment, sections of the liver were snap-frozen or placed in buffered-formalin, for blinded-histological evaluation of hematoxylin and eosin (HE) stained liver sections by a pathologist and determination of PMNs accumulation in the liver.

Plasma aspartate aminotransferase activity

Plasma AST activity was determined with a commercially available kit (#DG158K-U, Sigma Diagnostics, St. Louis, MO).

Histological analysis of liver injury

HE sections from formalin-fixed liver tissues obtained from sham-control and P-selectin Ab-treated livers were randomly selected and blindly analyzed for the degree of necrosis, hepatocellular vacuolization, glycogen depletion, zonal variations, and sinusoidal congestion, as measures of hepatic injury.

Polymorphonuclear leukocyte (PMNs) accumulation in the liver

PMNs accumulation in rat livers during perfusion was determined in formalin-fixed paraffin sections of the

liver obtained at each perfusion-sampling time point as previously described^[29]. A commercially available kit (91-C, Sigma-Aldrich, St. Louis, MO) was used to stain for sinusoidal-sequestered PMNs using the well-established Naphthol AS-D Chloroacetate esterase procedure, according to the manufacturer's directions. At least four random sections from each group were analyzed by viewing (blindly) fifty random high power fields (HPF, $\times 40$) on each section. Results were expressed as number of PMNs/50HPF.

RT-PCR analysis of Liver P-selectin and ICAM-1 mRNAs

Total RNA was extracted from liver tissue using an UltraSpec Total RNA Isolation Kit (#BL-10050, Biotecx Laboratories Inc., Houston, TX). Complementary DNA (cDNA) was transcribed with 4 μ g of total RNA, random hexamers, and a SuperScript II Preamplification System (#18089-011, GIBCO BRL, Life Technologies, Grand Island, New York) according to the manufacturer's protocol. Using specific primers for p-selectin ICAM-1, and GAPDH, their cDNAs were amplified by polymerase chain reaction (PCR) under the following conditions: P-selectin and ICAM-1 (35 cycles, 94°C for 60 s, 56°C for 60 s, and 72°C for 120 s), and GAPDH, (28 cycles, 94°C for 60 s, 52°C for 60 s, and 72°C for 60 s). PCR reaction primers (Sigma-Genosys, Woodlands, TX) used were as follows: P-selectin forward primer (5'-TGTATCCAGCCTCTTGGGCATTCC-3') and P-selectin reverse primer (5'-TGGGACAGGAAGTGA TGTTACACC-3') to give a 350-bp product; ICAM-1 forward primer (5'-AGGTGTGATATCCGGTAG-3') and ICAM-1 reverse primer (5'-TGGGACAGGAA GTGATGTTACACC-3') to give a 595-bp product; GAPDH forward primer (5'-GCCAAGTATGACAT CAA-3') and GAPDH reverse primer (5'-CCATATT CATTGTCATACCA-3') to give a 203-bp product. All PCR products were electrophoresed on a 2% agarose gel (Fisher Scientific, Fair Lawn, NJ). Bands were visualized by post staining for 30 min with GelStar Nucleic Acid Gel Stain (FMC Bioproducts, Rockland, MA), and photographed. Photographs were digitized and evaluated as stated above. The relative expression of P-selectin and ICAM-1 messenger RNAs (mRNA) were assessed by taking the ratio of the intensity of the DNA bands of P-selectin and ICAM-1 to GAPDH band, and expressed as arbitrary units. To ensure an equal amount of RNA was used for all samples, RNA concentration was determined spectrophotometrically, and its integrity evaluate on agarose gel. DNA bands were digitized (Corel Photohouse 2.0, Ontario, Canada) and evaluated using an image analysis software (Scion Image Beta 3b, NIH Image modified for Windows by Scion Corporation, Frederick MD).

Western blot analysis of liver P-selectin and ICAM-1 proteins

Homogenates and supernatants of liver samples were prepared as described by Vural *et al*^[30]. Proteins (P-selectin (14 μ g) and ICAM-1 (27 μ g)) in liver supernatants

were separated on 6% NuPAGE gels (Invitrogen, Carlsbad, CA) and transferred to nitrocellulose membranes (Schleicher and Schuel, Dassel). Equal transfer to membranes was confirmed by staining the membranes with Ponceau S (Aldrich-Sigma, St. Louis, MO). P-selectin bands were detected on membranes by incubating with an anti-P-selectin-specific rabbit primary antibody diluted 1:100 (CD62P, PharMingen, San Diego, CA). A horseradish peroxidase-conjugated anti-rabbit secondary antibody (sc-2350, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) diluted 1:1000 and an enhanced chemiluminescence (ECL) kit (Amersham Life Sciences, Piscataway, NJ) were used to visualize bands. ICAM-1 was detected on membranes by incubating with an anti-ICAM-1-specific mouse primary antibody (MCA1333R, Serotec, Raleigh, NC) diluted 1:50. A horseradish peroxidase-conjugated anti-mouse secondary antibody (Amersham Life Sciences, Piscataway, NJ) diluted 1:1000 was used to reveal ICAM-1 as described above. To ensure equal loading and normalize Western-blot bands of P-selectin and ICAM-1, membranes were also probed for β -actin. Membranes were immunoblotted for β -actin with an affinity purified goat polyclonal antibody diluted 1:500 (sc-1616, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and a horseradish peroxidase-conjugated anti-goat secondary antibody (sc-2350, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) (diluted 1:1000). Actin bands were visualized with a commercially available ECL kit, as stated above.

Liver total (GSH + GSSG) and oxidized (GSSG) glutathione levels

Liver GSH + GSSG and GSSG levels were determined by the method of Tietze^[31], as previously described by Jaeschke *et al*^[14].

Liver nuclear factor-kappa B (NF- κ B) activation

Activation of the redox-sensitive transcription factor NF- κ B in control and P-selectin Ab-treated livers was measured using a commercially available ELISA kit (Trans-AMTM NF- κ B p65 Transcription Factor Assay Kit, Active Motif, Carlsbad, CA). The assay was performed according to the manufacturer's procedure and as described by Renard *et al*^[32]. Nuclear proteins were extracted according to the procedure of Osarogiagbon *et al*^[33]. Approximately 100 mg of snap-frozen liver tissue was homogenized in 0.4 mL of cold TM buffer (10 mmol/L Tris-HCl, 1 mmol/L MgCl₂ (pH 7.0), containing completeTM protease inhibitors (Roche Diagnostics Corp., IN). Homogenates were centrifuged at 2000 r/min for 30 s, and the supernatant mixed with 200 μ L of lysis buffer, incubated at 4°C for 5 min, and centrifuged at 5000 r/min for 10 min. The nuclear pellets were reconstituted with lysis buffer, and centrifuged at 14000 r/min for 20 s at 4°C. Nuclear protein extract (15 μ g) of each sample was used to assay for NF- κ B activation. To ensure the specificity of the assay, the wild-type consensus oligonucleotide provided by the manufacturer served as a competitor to NF- κ B binding.

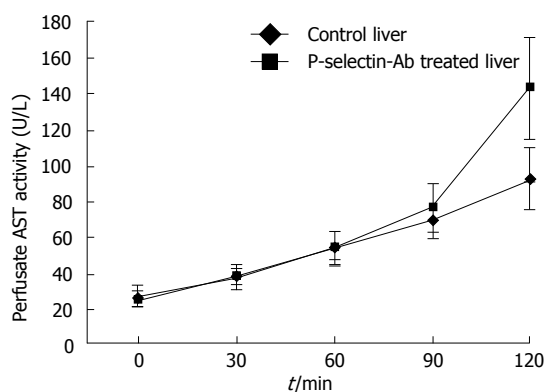


Figure 1 Perfusate aspartate aminotransferase (AST) activity in isolated-blood-perfused control and P-selectin-Ab treated rat livers after 6 h of cold ischemia.

Protein concentration

All protein concentrations were determined according to the method of Lowry *et al.*^[34].

Statistical analysis

Data were analyzed by unpaired Student's *t*-test or ANOVA. After ANOVA, results were subsequently subjected to Tukey's or Student-Newman Keuls non-parametric test to determined significant differences between groups.

RESULTS

Plasma AST activity, histologic analysis, and PMNs accumulation in the cold *ex vivo*-perfused liver

No significant difference in AST activity was found between control and P-selectin Ab-treated livers throughout the perfusion period (Figure 1). In addition, after 120 min of perfusion, similar degrees of point necrosis (solid arrows, Figure 2A, B) and inflammation (arrow heads, Figure 2C, D) were found in both cold *ex vivo*-perfused control and P-selectin Ab-treated livers. Similarly, no significant difference in hepatic PMNs accumulation was found between control and P-selectin Ab-treated livers after 120 min of perfusion (Figure 3).

Bile production in cold *ex vivo*-perfused liver

No significant difference in bile flow was found between control and P-selectin Ab-treated livers at individual sampling time-points throughout the perfusion period (data not shown). However, total bile production after 120 min of perfusion was significantly greater in P-selectin Ab-treated livers, compared to control (Figure 4).

RT-PCR analysis of P-selectin and ICAM-1 mRNAs in the cold-*ex vivo* perfused liver

No significant difference in P-selectin mRNA and protein was found between cold-*ex vivo* perfused control and P-selectin Ab-treated livers throughout the entire perfusion period (Figure 5A, B). In contrast, a significant reduction in ICAM-1 mRNA was found between cold-*ex vivo* perfused control and P-selectin Ab-treated livers at 60 min of perfusion (Figure 5A). However, although a

corresponding reduction in ICAM-1 protein was found, it did not reach statistical significance (Figure 5B). No significant difference in ICAM-1 mRNA and protein was found between cold *ex vivo*-perfused control and P-selectin Ab-treated livers at all other time points of the perfusion period (Figure 5).

Western blot analysis of P-selectin and ICAM-1 proteins in the cold-*ex vivo* perfused liver

No significant difference in P-selectin and ICAM-1 proteins was found between cold *ex vivo*-perfused control and P-selectin Ab-treated livers throughout the entire perfusion period (Figure 6A, B). Although P-selectin expression at 90 min of perfusion is clearly greater in control livers compared to Ab-treated livers, the intra-group variance precluded statistical significance.

GSH + GSSG and GSSG levels in the cold-*ex vivo*-perfused liver

No significant difference in GSH + GSSG and GSSG levels was found between cold *ex vivo*-perfused control and P-selectin Ab-treated livers throughout the entire perfusion period (Figure 7).

NF- κ B activation in cold *ex vivo*-perfused liver

No significant difference in NF- κ B activation was found between cold *ex vivo*-perfused control and P-selectin Ab-treated livers throughout the entire perfusion period (Figure 8).

DISCUSSION

Our study demonstrates that antibody-blockade of P-selectin alone failed to protect the rat liver from I/R injury in the IPRL model. However, as stated earlier, considerable evidence exist that suggests that P-selectin plays a major role in I/R injury^[18-21]. Existing reports also suggest that P-selectin mediates I/R injury of the liver by mediating initial rolling and margination of PMNs in the liver vasculature^[18-20]. However, other reports minimize its role in liver I/R injury, and its role in recruiting PMNs in the inflamed liver vasculature^[21-26].

At present, no convincing evidence exist for P-selectin expression on the mouse, human, and rat sinusoidal endothelia under normal and inflammatory conditions^[22,35,36], although a more recent study reported immunohistochemical evidence of P-selectin protein expression on the rat liver sinusoid endothelium after cold storage and orthotopic liver transplantation^[37]. In the present study, the accumulation of PMNs in livers after P-selectin blockade is probably due to physical trapping, resulting from the swelling of sinusoidal cells (e.g. endothelial and Kupffer cells), direct vasoconstriction, reduced deformability of PMNs exposed to activated complement factors, and the pre-existing intimacy (endothelium massaging) PMNs share with the sinusoidal endothelium, compared to the postsinusoidal endothelium. Once PMNs are slowed in the sinusoids, firm adhesion and transmigration may be

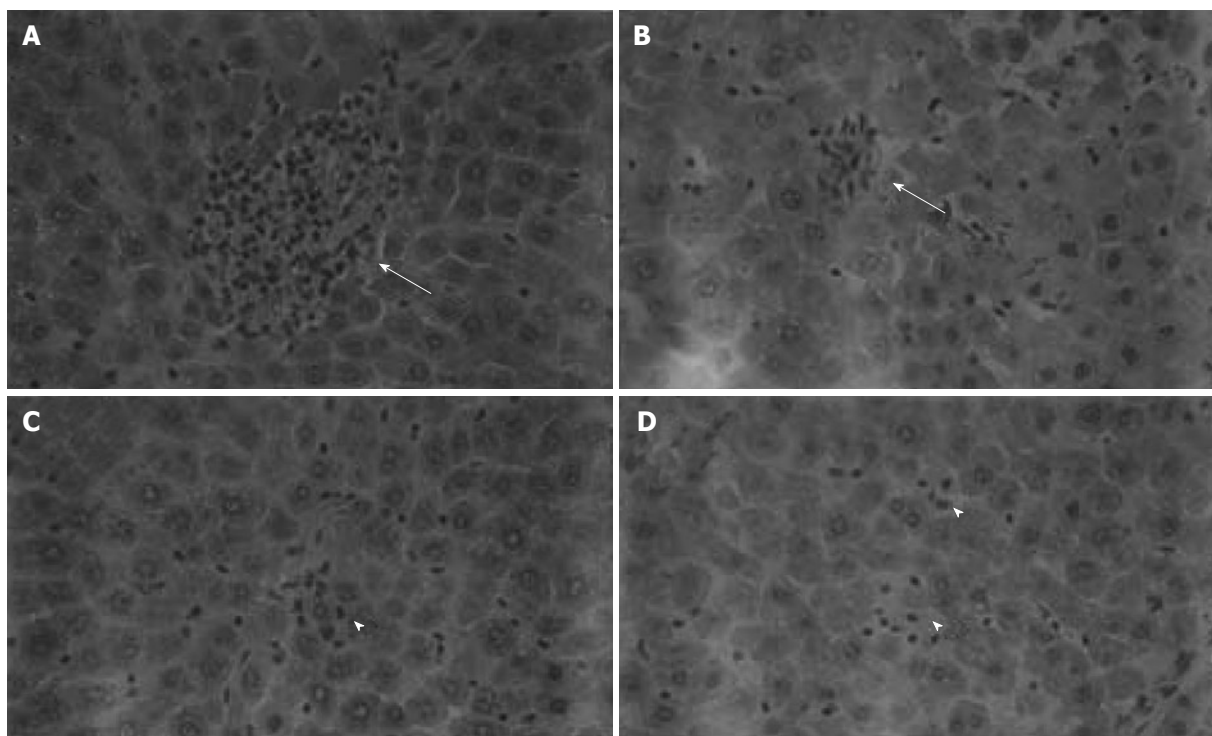


Figure 2 Histological analysis of isolated-blood-perfused control and P-selectin Ab-treated rat liver sections at 120 min perfusion after 6 h of cold ischemia (HE \times 400). A, B: Point necroses in livers of control and P-selectin Ab-treated livers at 120 min perfusion (solid arrows); C, D: Inflammation in both control and P-selectin Ab-treated livers at 120 min of perfusion (arrow heads).

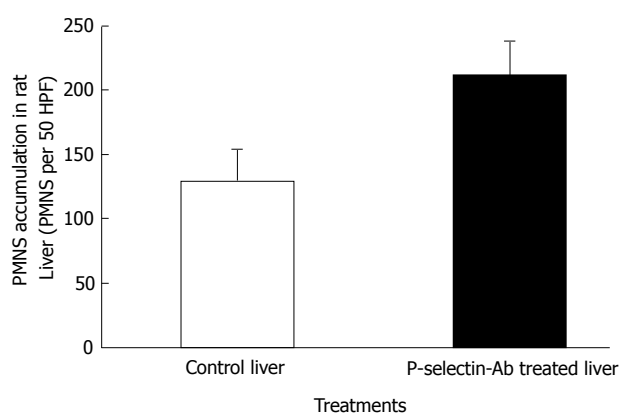


Figure 3 Accumulation of polymorphonuclear leukocytes (PMNs) in isolated-blood-perfused control and P-selectin Ab-treated rat livers at 120 min perfusion after 6 h of cold ischemia.

mediated by ICAM-1/ β_2 -integrins and VCAM-1/ β_1 -integrins^[23,38]. Alternatively, other, as yet unidentified, adhesion molecules may play a major role in the sequestration and transmigration of PMNs in liver sinusoids.

P-selectin blockade failed to protect the liver from I/R injury in our cold *ex vivo* model, as judged by the time dependent increase in AST activity, PMNs accumulation, and the similar histological-injury pattern found in control and P-selectin Ab-treated livers. Our findings are not in agreement with results of other studies that reported protection of the liver in cold *ex vivo* model with P-selectin blockade alone using the P-selectin ligands sPSGL-1 and rPSGL-Ig^[19,39] or an antibody to

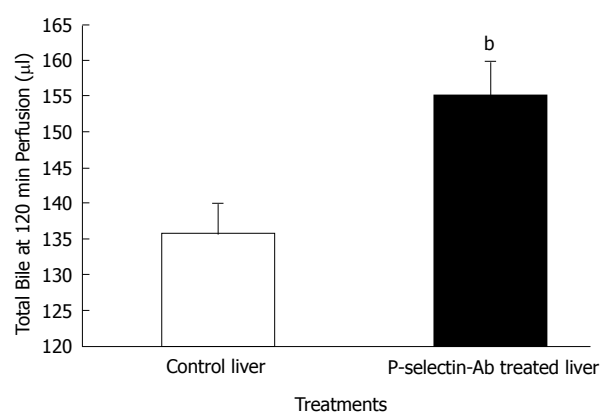


Figure 4 Bile production by isolated-blood-perfused control and P-selectin Ab-treated rat livers after 6 h of cold ischemia. ^b $P = 0.009$ vs control liver.

PSGL-1^[40]. However, P-selectin blockade did enhance total bile production by 120 min of perfusion, which agrees with earlier reports^[39,40]. Exactly how P-selectin blockade enhanced total bile production remains unclear.

P-selectin and ICAM-1 have been reported to be primary mediators of PMNs sequestration in the liver following I/R^[19,20,38,41]. However, in this study, P-selectin blockade had little or no effect on P-selectin and ICAM-1 mRNA and protein levels except for the significant decrease in ICAM-1 mRNA found at 60 min perfusion, which had a concomitant decrease in ICAM-1 protein that did not reach statistical significance. These results support our finding that P-selectin blockade did not significantly alter PMNs accumulation in the liver at 120 min perfusion. In fact, P-selectin blockade caused

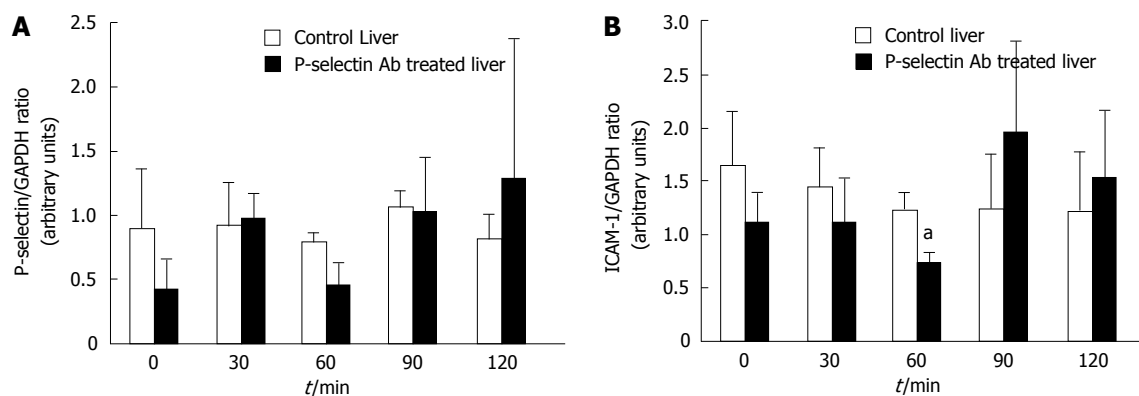


Figure 5 Semi-quantitative RT-PCR analysis of P-selectin and ICAM-1 mRNA levels in isolated-blood-perfused control and P-selectin Ab-treated rat livers after 6 h of cold ischemia. ^a $P < 0.05$ vs control liver.

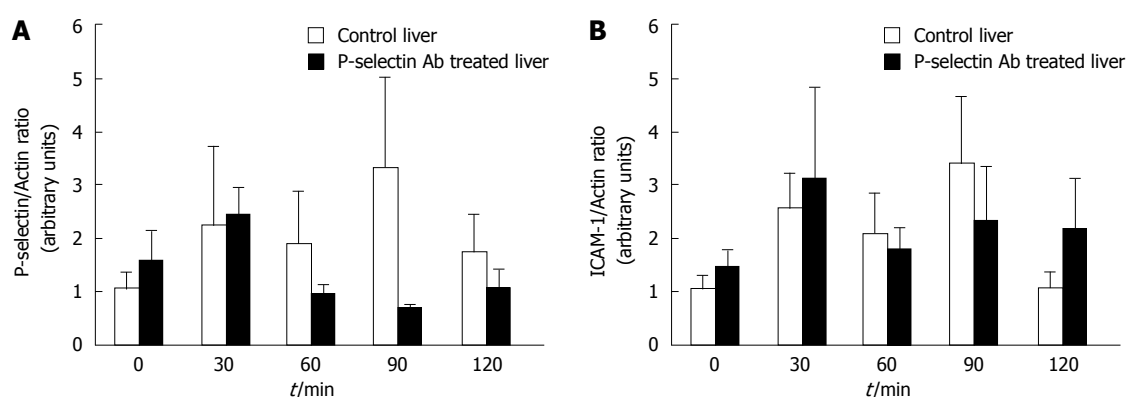


Figure 6 Western blot analysis of P-selectin and ICAM-1 protein levels in isolated-blood-perfused control and P-selectin Ab-treated rat livers after 6 h of cold ischemia.

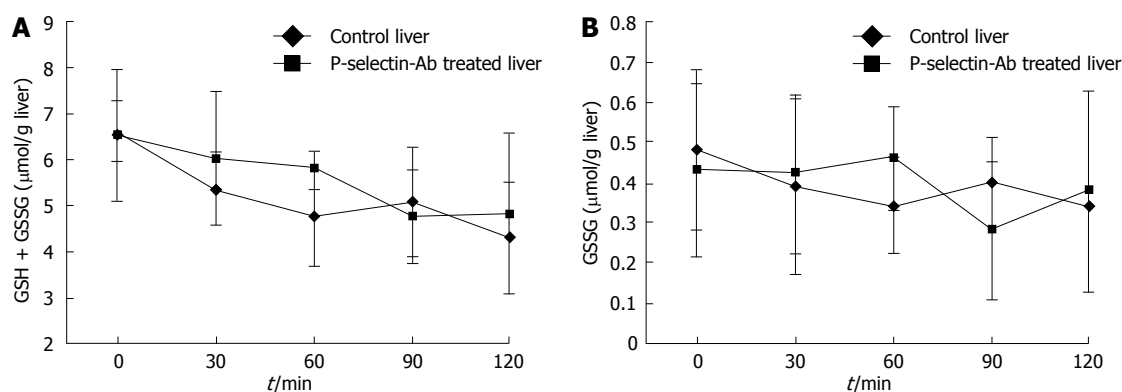


Figure 7 Reduced (GSH + GSSG) and oxidized (GSSG) glutathione levels in isolated-blood-perfused control and P-selectin Ab-treated rat livers after 6 h of cold ischemia.

an increase in PMNs accumulation in the liver, but not to a significant level. In addition, numerous studies have reported that ICAM-1 mediates firm adhesion and transmigration of PMNs in the liver following an inflammatory stimulus^[42-47]. However, some studies have questioned the absolute role of ICAM-1 in PMNs sequestration in hepatic vasculature^[21,48-52]. Furthermore, although the adhesion molecules ICAM-1 and VCAM-1 are expressed on cells lining the sinusoids, antibodies to selectins, integrins, and CAMs have all failed to prevent accumulation of PMNs in the liver sinusoids.

It is well documented that GSH plays a protective role in liver I/R injury^[1,53-57]. GSH can react with ROS such as hydrogen peroxide, peroxynitrite, and hypochlorous acid generated by KC and PMNs. We measured liver GSH and GSSG levels in our cold *ex vivo* model and found no significant difference with P-selectin-blockade treatment. If significant oxidative stress occurred during reperfusion, an increase in liver GSSG should have occurred^[1,53]. Although the major oxidative stress observed during perfusion is in the liver vasculature^[1,53], we did not measure GSSG levels in the

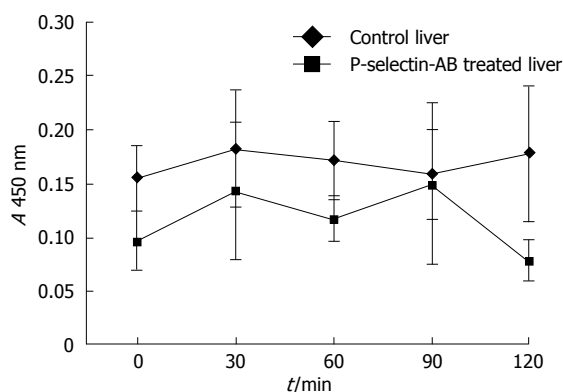


Figure 8 ELISA analysis of nuclear p65 as a measure of liver NF- κ B activation in isolated-blood-perfused control and P-selectin Ab-treated rat livers after 6 h of cold ischemia.

perfusate. Nevertheless, recent evidence from livers reperfused after cold storage has shown that hepatocytes may also be a source oxidative changes capable of impairing liver function during reperfusion^[58]. Therefore, in this study we should have detected any significant change in GSH or GSSG as an intracellular oxidative stress that occurred during reperfusion. The lack of significant change in liver total GSH and GSSG in our IPRL model may be taken as the absence/attenuation of oxidative stress in the liver^[53]. An alternate explanation for our failure to detect liver oxidative stress is that the above study used a more sensitive fluorescence detection compared to the colorimetric method employed in this study.

NF- κ B activation in the liver following I/R has been reported^[42,43,59]. Its activation has been reported as a requirement for I/R-dependent TNF- α induction in the liver^[59]. The inflammatory response following I/R of the liver is primarily mediated by cytokines (e.g. TNF- α , IL-1 β) and adhesion molecules P-selectin and ICAM-1, and VCAM-1. Induction of all the above inflammatory mediators requires activation of the transcription factor NF- κ B. In addition, existing evidence suggest that redox-sensitive transcription factors NF- κ B activation mediates the gene expression of pro-inflammatory cytokines such as TNF- α and IL-1 β ^[11]. Therefore, this study addressed the activation of liver NF- κ B in our cold *ex vivo* model, and failed to detect any significant reduction in liver NF- κ B activation with P-selectin blockade throughout the perfusion period. This finding supports our results for P-selectin and ICAM-1 expression, with the exception of ICAM-1 mRNA levels at 60 min perfusion. A corresponding decrease in NF- κ B activation was found at 60 min perfusion with P-selectin blockade, but did not achieve statistical significance.

Although most studies that characterized the benefit of P-selectin blockade in liver I/R injury used monoclonal antibodies or recombinant P-selectin ligands, in the present study we used a polyclonal antibody. Our use of a polyclonal antibody is not unusual, since other investigators have employed polyclonal antibodies to investigate blockade of mediators involved in I/R injury^[60]. It is likely that our

results are not in agreement with earlier studies because we employed a polyclonal antibody and at a different dose. Alternately, the antibody may have reacted with activated platelets and complement factors, as was later found for the monoclonal antibody PB1.3 used in the initial report that reported that P-selectin blockade alone protected the liver from I/R injury^[20]. Nonetheless, while the general mechanism of selectin-dependent rolling of PMNs followed by firmer adhesion to endothelial cells by integrins and ICAM-1 is applicable to the vasculature of some organs and tissues (heart, lung, intestine, and cremaster muscle), this might not be the case for the entire vasculature of the liver^[23,24].

In summary, this study demonstrates that while P-selectin blockade alone increased total bile flow in the IPRL model, it failed to protect the liver from I/R injury.

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Cytoreduction and hyperthermic intraperitoneal chemotherapy in the treatment of peritoneal carcinomatosis from pseudomyxoma peritonei

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Abstract

AIM: To investigate the most important aspects of hyperthermic intraperitoneal chemotherapy (HIPEC) that has been accepted as the standard treatment for pseudomyxoma peritonei (PMP), with special regard to morbidity, overall survival (OS) and disease free survival (DFS) over 10 years.

METHODS: Fifty-three patients affected by PMP underwent cytoreduction (CCR) and HIPEC with a "semi-closed" abdomen technique in our institution. The peritonectomy procedure and completeness of CCR were classified according to Sugarbaker criteria. Preoperative evaluation always included thoracic and abdominal CT scan to stage peritoneal disease and exclude distant metastases. Fifty-one patients in our series were treated with a protocol based on administration of cisplatin 100 mg/m² plus mitomycin C 16 mg/m², at a temperature of 41.5°C for 60 min. Anastomoses were always performed at the end of HIPEC. The mean duration of surgery was 12 h including HIPEC. Continuous monitoring of hepatic and renal functions and hydroelectrolytic balance was performed in the postoperative period.

RESULTS: Twenty-four patients presented with postoperative complications: surgical morbidity was observed in 16 patients and 6 patients were re-operated. All complications were successfully treated and no postoperative deaths were observed. Risk factors for postoperative morbidity were considered to be gender, age, body surface, duration of surgery,

Peritoneal Cancer Index (PCI) and tumor residual value (CC score). No statistically significant correlation was found during the multivariate analysis: only the CC score was statistically significant. The OS in our experience was 81.8%, with a DFS of 80% at 5 years and of 70% at 10 years.

CONCLUSION: In our experience, even if HIPEC combined with cytoreductive surgery involves a high risk of morbidity, postoperative complications can be resolved favorably in most cases with correct patient selection and adequate postoperative care, thus minimizing mortality. The association of CCR and HIPEC can be considered as the standard treatment for PMP. The OS and DFS results confirm the validity of this combined approach for the treatment of this rare neoplasm. The impact of preoperative chemotherapy on OS, in our opinion, is due to a major aggressiveness of tumors in treated patients.

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Key words: Peritonectomy; Pseudomyxoma peritonei; Hyperthermic perfusion; Hyperthermic intraperitoneal chemotherapy; Peritoneal carcinomatosis; Loco-regional treatment

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INTRODUCTION

Peritoneal carcinomatosis (PC) is one of the most common routes of dissemination of abdominal neoplasms; it may be present at the time of diagnosis of a primary tumor, but more frequently it arises as tumor recurrence after surgical treatment^[1].

PC is frequently associated with colorectal cancer, gastric cancer, ovarian carcinoma, and appendiceal cancer. Neoplasms with positive peritoneal cytology

show high rates of peritoneal dissemination^[2-6].

Pseudomyxoma peritonei (PMP) is a rare condition, with an incidence of 1/1 000 000 per year, characterized by copious mucus (so-called “jelly belly”) containing rare epithelial cells. According to Ronnet, PMP was histologically classified into disseminated peritoneal adenomucinosis (DPAM), peritoneal mucinous carcinomatosis (PMCA) and an intermediate or discordant feature group (ID)^[7]. Recent studies show that most cases of PMP originate from ruptured appendiceal tumors with progressive dissemination in the peritoneal cavity of mucin-producing epithelial-cells^[8,9]. Lymph-nodal or hematogenous metastases are rare in PMP and evidence suggests it has a poor prognosis.

PC from PMP is generally considered a lethal disease, with a limited response to conventional chemotherapeutic treatments^[10]. While systemic chemotherapy has little impact on the treatment of peritoneal disease, some centers have reported encouraging results with intraperitoneal hyperthermic chemoperfusion (HIPEC)^[11,12].

This technique is based on surgical cytoreduction (CCR) of the primitive cancer, peritonectomy and HIPEC. The principle of locoregional treatments is to obtain an elevated and persistent drug concentration for the tumor, with a limited systemic concentration. Many studies reported an impact on overall survival (OS) and disease-free survival (DFS) in patients affected by carcinomatosis of mucinous cancers such as PMP^[13,14] and in recent trials this combined approach has been proposed as standard treatment for PMP.

In this study we report the results of a 10-year experience with this type of treatment in our institution, where 53 patients with PMP were treated with CCR and HIPEC, with special reference to follow-up and risk factors for postoperative complications.

MATERIALS AND METHODS

For the present study, 53 patients (23 male and 30 female, mean age 58 years, range 32-72) with PMP who underwent surgical treatment and HIPEC between October 1998 and June 2008 at the Department of General Surgery and Surgical Oncology, San Giuseppe Hospital, were considered. Preoperative evaluation always included thoracic and abdominal CT scan to stage peritoneal disease and exclude distant metastases; upper digestive endoscopy and colonoscopy generally completed tumor staging. A careful preoperative evaluation of the patient's general condition was always performed, and included complete blood tests, electrocardiogram, cardiac ultrasound, and spirometry. The presence of hepatic or extra-abdominal metastases, poor general condition or performance status > 2 according to the Eastern Cooperative Oncology Group (ECOG) and an age of > 72 years were generally considered contraindications to the treatment. Informed consent was obtained from all patients^[15].

Just after laparotomy, a complete intraoperative staging of peritoneal disease was performed using the peritoneal cancer index (PCI)^[16]; the mean PCI was 22.

Surgical technique

The peritonectomy procedure was classified and performed according to Sugarbaker's criteria: (1) Central peritonectomy consists of the removal of previous scars, greater omentectomy (performed by stripping the superficial peritoneal layer of the transverse mesocolon) and a close dissection to the greater curvature of the stomach. Sometimes splenectomy could be necessary *en bloc* with the greater omentum and the left diaphragmatic peritoneum; (2) Left upper quadrant peritonectomy consists of the stripping of the peritoneal tumor tissue from beneath the left hemidiaphragm, left adrenal gland, distal portion of the pancreas, and the cephalad half of Gerota's fascia; (3) Right upper quadrant peritonectomy consists of right hemidiaphragmatic peritoneal stripping, removal of tumor from the right subhepatic space and from the surface of the liver by the stripping of the Glisson's capsule. Peritonectomy is concluded with the removal of the peritoneum covering the right kidney and Morrison's pouch; (4) Lesser omentum peritonectomy is performed after the cholecystectomy, and in this procedure the cancerous tissue which covers the common duct and hepatic artery is stripped from the base of gall bladder bed towards the duodenum. This phase is concluded by the stripping of omental bursa; (5) Pelvic peritonectomy with *en bloc* removal of pelvic peritoneum, sigmoid colon, rectum, uterus and salpingo-oophorectomy; (6) Peritonectomy of the lateral abdominal wall. Implants on the visceral serosa are removed by electrosurgical local dissection and the peritonectomies are variously combined with resections of viscera involved in tumor (total gastrectomy or total colectomy).

The completeness of CCR was also classified according to Sugarbaker's criteria^[17] as: CCR-0 (no residual tumor) in 35 cases, CCR-1 (no residual nodule greater than 2.5 mm in diameter) in 18 cases, CCR-2 (no residual nodules greater than 25 mm) in none of the cases and CCR-3 (residual nodules greater than 25 mm) in none of the cases.

HIPEC was performed according to the “semi-closed” abdomen technique^[18]. Five drain tubes are placed in the abdominal cavity. There are 2 inflow tubes, and they have multiple holes. They present 2 diffusion lines for the homogeneous distribution of drugs into the abdominal cavity (1 in the sovramesocolic branch, 1 in the pelvis). Three outflow tubes are placed respectively in the pelvis and in the subdiaphragmatic spaces. Backhaus forceps are used to close the cranial and caudal portion of abdominal wound. The skin is then suspended by a self-retaining retractor, placed at more or less 15 cm from the abdomen, by plastic self-blocking strings. This kind of placement creates the virtual cavity needed to perform HIPEC. The central portion of the wound is suspended by the retractor too and covered with a laparoscopic device with sterile drapes on it, with a hole in the middle. The drain tubes are connected to a perfusion system formed by 2 pumps and a heat exchanger to heat the perfusion liquid. The inflow and

outflow pumps are connected through a reservoir, so it is possible to achieve continuous circulation of the perfusate at the speed of more or less 1 L/min. The pumps are controlled by a computerized system that allows the checking of the flow rate and the temperature of the heat exchanger. Three intraperitoneal temperatures are checked by probes; the inflow temperature, outflow temperature, and the patient esophageal temperature. The amount of circulating perfusate required (solution for peritoneal dialysis) is calculated according to the patient's body surface. During perfusion, the surgeon mixes the perfusate by hand through the hole in the sterile drapes. When the ideal intraperitoneal temperature is reached, the drugs are added to the circuit and HIPEC is performed for 60 min. Fifty-one patients in our series were treated with a protocol based on administration of cisplatin 100 mg/m² plus mitomycin C 16 mg/m², at a temperature of 41.5°C. Two patients were treated with mitomycin C 35 mg/m² for 60 min at a temperature of 40.5°C, according to the Netherland protocol, because of significant side effects from preoperative systemic chemotherapy with platinum. Anastomoses were always performed at the end of HIPEC. The mean duration of surgery was 12 h including HIPEC (range 8-16 h). At the end of the operation, the patient was admitted to the intensive care unit, and then returned to the surgical department when cardiovascular and pulmonary functions became stable. Continuous monitoring of hepatic and renal functions and hydroelectrolytic balance were performed afterwards. The primitive neoplasm was an appendicular adenocarcinoma in 37 patients (69.8%) and an appendicular adenoma in 16 patients (30.2%). Twenty-one patients (39.6%) with histological diagnosis of appendicular adenocarcinoma had been treated with systemic chemotherapy before our operation. Because of the massive involvement of viscera and peritoneum, in some selected patients we performed the treatment in steps. Three patients were treated in 2 steps, and 1 patient was treated in 3 steps. In these cases, we performed the upper abdominal CCR in the first step, then the patient was submitted to systemic chemotherapy for 2 or 3 mo. The second step consisted of lower abdominal CCR and peritoneal perfusion of the entire peritoneal cavity. The details of the CCR procedures are displayed in Table 1.

Statistical analysis

In this study the statistical analyses focused on postoperative complications: the histopathological, clinical and follow-up data were stored in a database. The presence of postoperative complications was considered as the dependent variable whereas gender, age, body mass index, primary tumor, previous systemic chemotherapy, operative time, stage of PCI, and CCR were covariates.

Multivariate analysis of factors was performed by the Cox proportional hazard model. OS was dated from the day of surgery to the time of death due to any causes; progression-free survival (PFS) was dated from the day of the surgery to the time of postoperative disease

Table 1 Extent of CCR and HIPEC in 53 patients affected by PMP

Procedures	No.
Greater omentectomy	48
Left colectomy	30
Right colectomy	34
Total abdominal hysterectomy	16
Bilateral salpingo-oophorectomy	20
Splenectomy	40
Cholecystectomy	34
Small bowel partial resection	27
Total gastrectomy	5
Sub-total gastrectomy	3
Distal pancreatectomy	6
Righth upper quadrant peritonectomy	47
Left upper quadrant peritonectomy	38
Pelvic peritonectomy	43

progression. The survival curves for both OS and PFS were calculated according to the Kaplan-Meier method. The log-rank test was used to assess the significance of the comparison between survival curves.

The Statistical Package for the Social Sciences software (version 11.0) (SPSS, Chicago, IL, USA) was used for statistical analysis: $P < 0.05$ was considered significant.

RESULTS

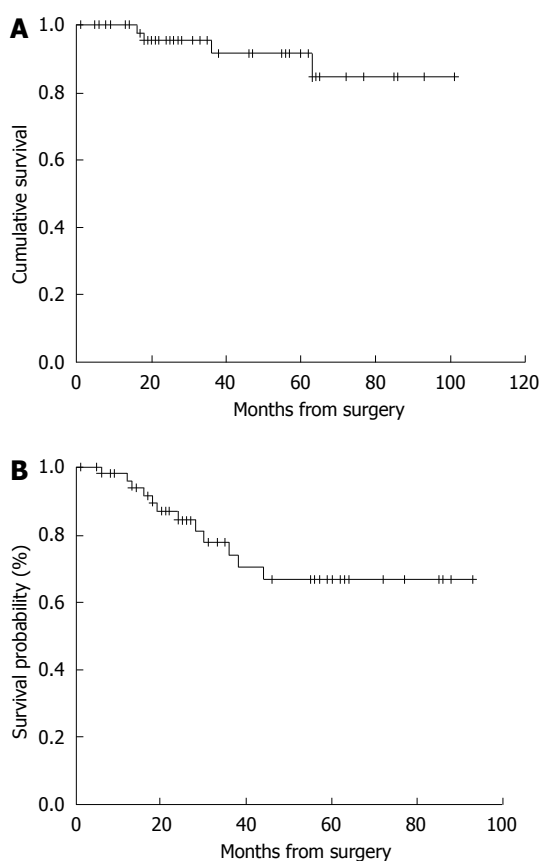
At the end of follow-up of the 53 patients, 5 and 10 year OS was 94% and 84.6%, respectively (Figure 1A). DFS was 80% and 70% at 5 and 10 years, respectively (Figure 1B).

OS according to the PCI, completeness of CCR (CC-score), histological type, and pre-operative chemotherapy (done *vs* not done) are shown in Figure 2A-D. At the time of the present analysis 48 patients are alive without disease. Two patients died due to systemic disease progression at 16 and 63 mo, respectively, after the operation; 3 patients are alive with disease and intraperitoneal relapse but are not undergoing a further operation, with follow-up of 57, 28, 24, 19, 10 mo, respectively. Three patients had intraperitoneal relapse and were treated with tumor resection followed by HIPEC: 1 of those patients is alive without disease 17 mo after the second surgical procedure; the other 2 patients were treated only with CCR and they are alive without disease after 24 mo of follow-up.

For calculation of the morbidity rate, we considered postoperative complications occurring during the hospital stay or within 30 d of surgery. In 24 patients (45%) we observed postoperative complications: surgical morbidity was observed in 16 patients (3 intestinal fistulas, 2 urinary tract perforations, 2 abdominal abscesses, 4 wound infections, 1 prolonged ileus, 2 postoperative haemorrhages, 1 abdominal wall dehiscence, 1 bleeding from a gastric ulcer) and medical complications were observed in 8 cases (1 arrhythmia, 3 grade 2 hematological toxicities, 1 acute renal failure, 1 cutaneous rash, 2 cases of sepsis). Six patients were

Table 2 Association between morbidity and clinical variables *n* (%)

Variable	No. of cases	With complications	Without complications	P-value
Gender				NS
Male	23	11 (46)	12 (41)	
Female	30	13 (54)	17 (59)	
Age (yr)		59 ± 10	56 ± 9	NS
Body mass index (Kg/m ²)		25.4 ± 5.3	26.8 ± 4.7	NS
Previous systemic chemotherapy				NS
Performed	21	10 (42)	11 (38)	
Not performed	32	14 (58)	18 (62)	
PCI				NS
> 16	36	18 (75)	18 (62)	
< 16	17	6 (25)	11 (38)	
Operative time (h)		8.5 ± 3.0	7.1 ± 2.1	NS
Completeness of cancer resection				0.017
CCR-0	35	12 (50)	23 (79)	
CCR-1	18	12 (50)	6 (21)	

**Figure 1** 53 cases of PMP. A: Overall survival (Kaplan-Meier); B: Disease free survival (DFS).

re-operated and 1 patient underwent ureteric stenting. One patient with abdominal abscess was submitted to ultrasound-guided drainage and 1 patient with bleeding from a gastric ulcer was treated by endoscopic haemostasis. All other complications were successfully treated by medical therapy (Table 2). No postoperative deaths were observed. An analysis of risk factors for postoperative morbidity rate was performed. Gender, age, body surface, duration of surgery, PCI and tumor residual value were considered to be risk factors. No statistically significant correlation between the

Table 3 Postoperative complications observed in 24 patients. Multiple complications are included

Complication	No. of cases	Treatment (No. of cases)
Surgical		
Wound infection	4	Drainage
Urinary tract perforation	2	Reoperation (1); Urinary stenting (1)
Intestinal fistula	3	Reoperation
Abdominal abscess	2	US-guided drainage (1); medical (1)
Prolonged ileus	1	Medical
Bleeding from gastric ulcer	1	Endoscopic haemostasis
Intraabdominal bleeding	2	Reoperation
abdominal wall dehiscence	1	Conservative
Medical		
Grade ≥ 2 hematological toxicity	3	Medical
Acute renal failure	1	Medical
Arrhythmias	1	Medical
Cutaneous rash	1	Medical
Sepsis	2	Medical

analyzed variables and the incidence of postoperative complications was found except for CC-score ($P < 0.017$) (Table 3).

Final follow-up data from our experience indicated that survival probability may be good in patients with histological type appendicular adenoma who are optimally cytoreduced (CC-0). An interesting relief was related to whether preoperative chemotherapy was performed or not.

DISCUSSION

HIPEC associated with cytoreductive surgery is becoming a widely accepted procedure for the treatment of PMP.

Like reports in several studies, the results of our experience indicate that, even when combined with an aggressive surgical procedure, HIPEC is associated with an acceptable risk of postoperative complications and mortality^[19-24]. The incidence of postoperative complications was similar to that of other reports^[25-29]

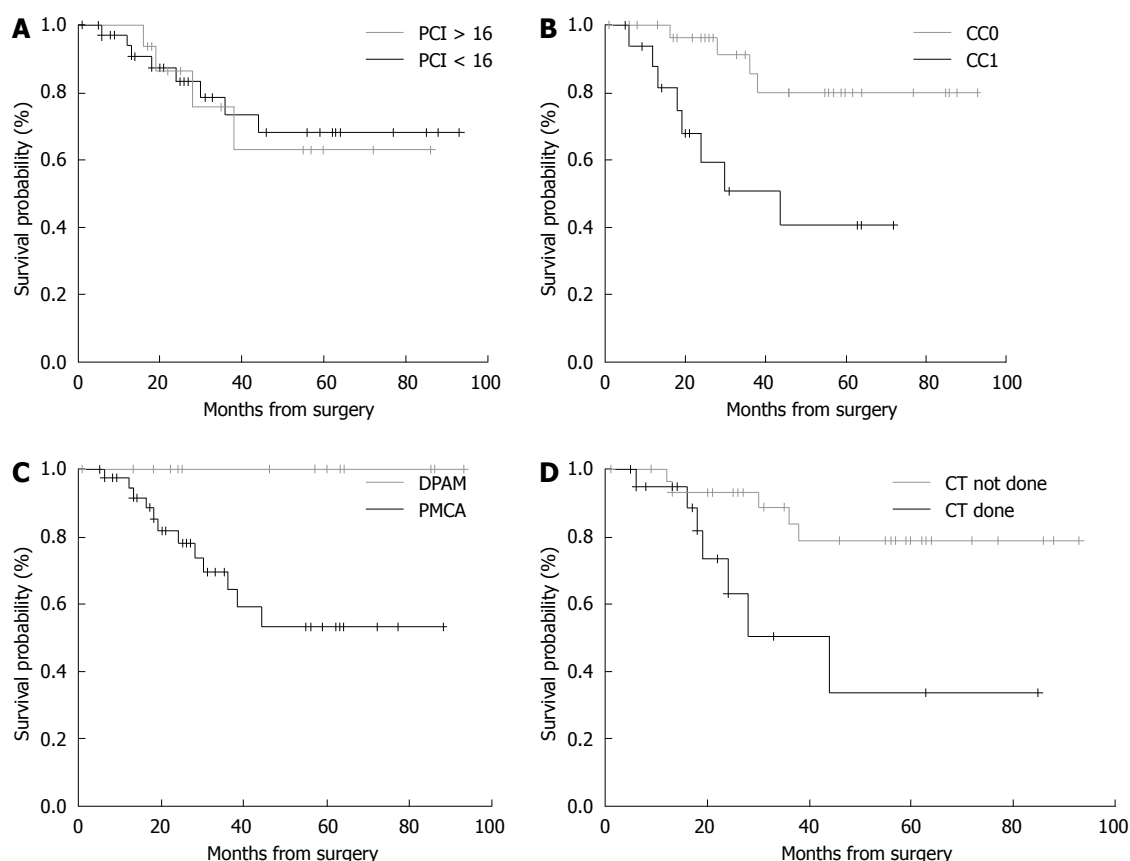


Figure 2 Overall survival (OS) in 53 cases of PMP. A: According to PCI status (P N.S.); B: According to the completeness of CCR ($P < 0.003$); C: According to the histological tumor type ($P < 0.014$); D: According to previous systemic chemotherapy ($P < 0.034$).

and major morbidity occurred in 45% of patients, which is also similar to other recent experiences^[27,29,30]. All the complications were successfully treated with surgical or medical therapy. We believe that careful preoperative selection of patients, and adequate postoperative monitoring and care are crucial in order to minimize the incidence of postoperative complications in these patients.

The advanced stage of neoplastic disease and immunodeficiency status of patients previously subjected to chemotherapy were important factors that probably contributed to the occurrence of septic complications after an extended surgical procedure. In our series 1 patient underwent ultrasound-guided drainage of an abdominal abscess and in the others septic complications were successfully treated with medical therapy.

Intestinal fistula has been reported to be an important cause of morbidity and mortality in patients submitted to HIPEC, with an incidence rate ranging from 6% to 27%^[21-23, 31-33]. Younan *et al*^[32] reported that male gender, duration of surgery, and no previous systemic chemotherapy were independent predictors of bowel complications. The direct effect of HIPEC even in non-resective procedures can be associated with intestinal fistulas in the postoperative period^[33,34]. We always performed anastomotic suture following HIPEC but in our series the 3 cases of intestinal fistula were due to intestinal perforation not involving anastomosis.

Recent studies have reported the duration and extent

of surgery, visceral resections, PCI and incomplete CCR to be important risk factors for postoperative complications^[21,26,31]. In our patients, we did not find this correlation, probably because of the limited number of cases. PCI > 16 was an independent predictor of postoperative morbidity only at univariate analysis and seemed to have an impact on the complication rate, but not on OS.

The largest series of PMP undergoing combined treatment was reported by Sugarbaker^[35]: in this series completeness of CCR and Ronnet's criteria were the most important factors correlated with survival and morbidity^[36].

Complete CCR was obtained in most of our patients. In our series, we didn't have patients with an elevated CC score (CC-2 or CC-3): as a consequence, even though the morbidity rate was high we did not find a correlation between cytoreductive status and complication rate. However, the CC-score in our experience was strictly correlated to DFS with an evident result between CC-0 and CC-1 patients ($P < 0.003$).

The limited number of patients in our series did not allow further stratifications and for these reasons, the potential impact of other factors on morbidity cannot be excluded.

Survival data indicate that high long-term survival could be achieved in patients with histological type DPAM *vs* PMCA ($P < 0.014$). Furthermore in the present series the adverse prognostic value of preoperative systemic

chemotherapy was a unexpected finding that may be not easy to explain: the patient that received chemotherapy had a poor prognosis compared to those that did not undergo chemotherapy ($P < 0.034$). The same evidence was observed in the series of Baratti *et al*^[37].

The hypothesis was that after chemotherapy, mucinous appendiceal tumors change to a more invasive process: it is quite possible that differences in chemotherapy penetration of mucinous and solid tumours may result in persistence and progression of the more solid components of a non uniform tumor. Appendiceal tumors are described as having large areas of adenomucinosi with small, even minute, areas of more aggressive tissue: the penetration of chemotherapy drugs into the mucin that contains adenomatous epithelial cells may eradicate these cells but the small foci of solid tumor may not be completely penetrated by chemotherapy. It is possible that this process selected resistant and more aggressive tumor cell clones but the explanation for the poor results of the treatment in these patients requires further investigation.

In conclusion the present study confirms that an aggressive approach can improve survival in selected patients with PMP. Although HIPEC combined with CCR has a high risk of morbidity, postoperative complications could be resolved favorably in most cases with correct patient selection and adequate postoperative care, thus minimizing mortality. Residual tumor (CC), preoperative chemotherapy and histological type PCMA significantly influence the prognosis of these patients^[20,37].

To improve this encouraging survival outcome, it is very important to unify the surgical experience of expertise centers and adequate patient selection. Our results suggest also the need of an integrated approach to this rare neoplasm to identify the biological aspect of PMP that influences the prognosis and the evolution of the disease.

COMMENTS

Background

Peritoneal carcinomatosis is generally considered a lethal disease, with a mean survival time of 6 mo after conventional chemotherapeutic treatments. Systemic chemotherapy has little impact on treatment of peritoneal disease, but some centres have reported encouraging results with intraperitoneal hyperthermic chemoperfusion (HIPEC). Locoregional treatments are considered a new frontier in the management of this condition: it is possible to achieve an elevated and persistent drug concentration in the tumor, with limited systemic effects. Many studies reported an impact on overall survival and disease-free interval in patients affected by carcinomatosis, of mucinous cancers such as pseudomyxoma peritonei (PMP) and in recent trials this combined approach has been proposed as standard treatment for PMP.

Research frontiers

The present study confirms that an aggressive approach can improve survival in selected patients with PMP. Although HIPEC combined with cytoreductive surgery involves a high risk of morbidity, postoperative complications can be resolved favorably in most cases with correct patient selection and adequate postoperative care, thus minimizing mortality. Residual tumor (CC), preoperative chemotherapy and histological type of PMP can be considered as independent variables able to significantly influence the prognosis of these patients. To improve this encouraging survival outcome, it is very important to

unify the surgical experience of expertise centres. Our results suggest also the need for an integrated approach to this rare neoplasm to identify the biological aspects of PMP that influence the prognosis and the evolution of the disease.

Innovation and breakthroughs

In this paper we report a very important proof on the integrated approach to PMP. This lethal disease can be treated with good results: in fact 5 and 10 year overall survival was, respectively, 94% and 84.6% in our experience and disease free survival was 80% and 70% at 5 and 10 years, respectively.

Application

On future application, the end point of this approach would be to improve a standard treatment for this particular disease to reduce the surgical risk of major complications. Correct patient selection and adequate postoperative care may minimize the considerable complication rate that is very high (45%).

Peer review

This is a very interesting study on pseudomyxoma peritonei and its treatment with intraperitoneal hyperthermic chemoperfusion.

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RAPID COMMUNICATION

Effect of propranolol on the splanchnic and peripheral renin angiotensin system in cirrhotic patients

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RESULTS: PRA, Ang I, Ang II and Ang-(1-7) were significantly lower in the portal vein and periphery in all subgroups treated with propranolol as compared to non-treated. The relationships between Ang-(1-7) and Ang I levels and between Ang II and Ang I were significantly increased in LD group receiving propranolol. The ratio between Ang-(1-7) and Ang II remained unchanged in splanchnic and peripheral circulation in patients under β -blockade, whereas the relationship between Ang II and Ang I was significantly increased in splanchnic circulation of LT patients treated with propranolol. During liver transplantation, cardiac output and index as well systemic vascular resistance and index were reduced in propranolol-treated subgroup.

CONCLUSION: In LD group, propranolol treatment reduced RAS mediators, but did not change the ratio between Ang-(1-7) and Ang II in splanchnic and peripheral circulation. Furthermore, the modification of hemodynamic parameters in propranolol treated patients was not associated with changes in the angiotensin ratio.

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Key words: β -blockade; Cirrhosis; Renin angiotensin system; Angiotensin-(1-7)

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Abstract

AIM: To evaluate the effect of β -blockade on angiotensins in the splanchnic and peripheral circulation of cirrhotic patients and also to compare hemodynamic parameters during liver transplantation according to propranolol pre-treatment or not.

METHODS: Patients were allocated into two groups: outpatients with advanced liver disease(LD) and during liver transplantation(LT). Both groups were subdivided according to treatment with propranolol or not. Plasma was collected through peripheral venipuncture to determine plasma renin activity(PRA), Angiotensin(Ang) I, Ang II, and Ang-(1-7) levels by radioimmunoassay in LD group. During liver transplantation, hemodynamic parameters were determined and blood samples were obtained from the portal vein to measure renin angiotensin system(RAS) components.

INTRODUCTION

The Renin-Angiotensin System(RAS) is a multilayered complex system. Previously, Angiotensin (Ang) II was thought to be the principle active peptide, exerting its action through type I and type II receptors^[1]. However, our understanding of the RAS has significantly

grown^[1,2]. Additional active RAS peptides have been identified such as Ang III, with actions similar to Ang II and Ang IV, which exerts its activity at insulin-regulated amino peptidase receptors, and Ang-(1-7), which acts mainly through Mas receptors^[1,2]. There is evidence that the RAS acts at the local tissue and even intracellular level through specific receptors by exerting paracrine, endocrine and intracrine functions^[1,2]. Further, the system mediates a variety of opposing physiological actions including vasoconstriction/vasodilation, fibrosis/antifibrosis and inflammation/anti-inflammatory^[1,2]. Therefore, the RAS is now viewed as a dual system composed of two arms: a vasoconstrictor arm formed by angiotensin converting enzyme(ACE)-Angiotensin (Ang) II -AT1 receptor and a vasodilator arm with ACE2-Ang-(1-7)-Mas receptor. The ACE2-Ang-(1-7)-Mas arm mainly acts as a counter-regulatory mechanism for the vasoconstrictor arm^[1]. According to this novel concept, the final functional effect of the RAS may reflect a balance between these two arms^[2-5].

This novel view of the RAS makes the evaluation of this system in cirrhosis particularly challenging. In this regard, recent studies have suggested that the RAS seems to be involved in cirrhosis through its two main arms: the first (ACE-Ang II -AT1) by inducing liver fibrosis^[6] and maintaining the basal vascular tonus in cirrhosis^[7] and the second [ACE2-Ang-(1-7)-Mas] by exerting an anti-fibrotic role^[8,9] and probably by participating in the vasodilation of cirrhosis^[10].

Non-selective β -adrenergic blockers have been widely used in treatment of portal hypertension in cirrhosis. β -blockers lower portal pressure by reducing portal blood flow as a consequence of a decreased cardiac output (β_1 -receptor blockade) and arteriolar splanchnic vasoconstriction (β_2 -receptor blockade)^[11]. β -blockers also inhibit renin secretion^[12]. However, the effect of propranolol on RAS mediators has still not been quantified, and neither have the hemodynamic changes that might occur during liver transplantation in cirrhotic patients pre-treated with propranolol. Since non-specific β blockade has been a standard approach to controlling the symptoms of portal hypertension and because the RAS seems to influence the outcome of portal hypertension and cirrhosis, it is reasonable to ask if there is a functional relationship between the RAS and beta-receptor system. For this purpose, we have taken in this study the first steps to understand how β_1 and β_2 blockade affects the RAS in cirrhotic patients. Thus, the aim of the present study was to compare the levels of plasma renin activity (PRA), Ang I, Ang II and Ang-(1-7), measured in the splanchnic and peripheral circulations of cirrhotic patients receiving or not propranolol and to evaluate the effect of previous administration of propranolol on hemodynamic parameters during liver transplantation.

MATERIALS AND METHODS

Patients

This cross-sectional study used a convenience sample

Table 1 Clinical characteristics and casual measurements of advanced liver disease outpatients (LD) treated with propranolol or not

Characteristics and measurements	LD with propranolol (n = 9)	LD without propranolol (n = 7)
Age (yrs)	45 \pm 2	54 \pm 5
Sex male/female	5 (55.6%)/4 (44.4%)	4 (57%)/3 (43%)
Child Pugh Score	9.8 \pm 0.5	11.0 \pm 0.8
MELD Score	27.1 \pm 1.3	29.3 \pm 2.1
Albumin (g/dL)	2.7 \pm 0.2	2.4 \pm 0.3
Bilirubin (mg/dL)	2.5 (1.3-5.1)	2.5 (1.2-7.1)
Creatinine (mg/dL)	1.2 (0.8-2.3)	1.0 (1.0-1.45)
INR (International Normalized Ratio)	1.62 (1.01-6.15)	1.55 (1.20-2.20)
Serum Na ⁺ (mEq/L)	133.0 \pm 1.6	126.0 \pm 2.7 ^a

Data are expressed as mean \pm SE or median (25 and 75 percentile), except for sex where number of patients and percentages are shown. ^a*P* < 0.05 for the comparison of LD with propranolol and LD without propranolol (unpaired *t* test for mean comparisons and Mann-Whitney test for median comparisons).

Table 2 Clinical characteristics and casual measurements of patients undergoing liver transplantation (LT) pre-treated with propranolol or not

Characteristics and measurements	LT with propranolol (n = 10)	LT without propranolol (n = 11)
Age (yr)	50.6 \pm 3.4	50.0 \pm 2.6
Sex male/female	3 (30%)/7 (70%)	7 (63.6%)/4 (36.4%)
Child Pugh Score	10.5 \pm 0.4	11.2 \pm 0.6
MELD Score	28.0 \pm 1.1	29.8 \pm 1.6
Albumin (g/dL)	2.81 \pm 0.09	2.61 \pm 0.15
Bilirubin (mg/dL)	3.38 \pm 1.20	3.70 \pm 0.87
Creatinine (mg/dL)	1.0 (1.0-1.45)	1.05 (0.75-1.50)
INR (International Normalized Ratio)	1.36 (1.32-1.95)	1.69 (1.24-2.11)
Serum Na ⁺ (mEq/L)	135.2 \pm 1.0	130.1 \pm 1.8 ^a

Data are expressed as mean \pm SE or median (25 and 75 percentile), except for sex where number of patients and percentages are shown. ^a*P* < 0.05 for the comparison of LT with propranolol and LT without propranolol (unpaired *t* test for mean comparisons and Mann-Whitney test for median comparisons).

recruited from either the Alfa Institute of Hepatology/Liver Transplantation or the Clinical Primary Care Center of our institution.

Inclusion criteria

Patients diagnosed with hepatic cirrhosis defined through liver histopathology and/or ultrasonography findings were included in this study. Tables 1 and 2 display the Child-Pugh^[13] and MELD^[14] scores of our patients (all patients were on the waiting list for liver transplantation). The etiology of the liver disease was established in the majority of the subjects (69%), and included alcoholism, virus C, virus B and bile cirrhosis. The cirrhotic patients were allocated to two study groups: one group was composed of patients who had advanced liver disease and were seen in an outpatient clinic (LD, *n* = 16) and the second group was composed of liver transplant recipients during surgery (LT, *n* = 21). Each

of these two groups was further divided into patients who received propranolol and those who did not. The assistant physician was the only person responsible for the prescription and indication of propranolol treatment and the study protocol did not interfere with any medical prescriptions and recommendations. Thus, patients who were already on treatment with propranolol were then compared to those that did not receive treatment. As shown in Tables 1 and 2, the two subgroups of patients (treated *vs* non-treated) are comparable in the major demographic characteristics.

The LD group comprised outpatients with ascites and extra-hepatic complications such as encephalopathy and moderate to large esophageal varices (> 5 mm) with risk of bleeding. These patients were using diuretics (furosemide: 40-80 mg/d associated with spironolactone: 25-100 mg/d). Nine of these patients were also receiving propranolol for a mean period of 60 d (40-80 mg/d). The doses of propranolol were titrated to achieve a 20%-25% change in baseline heart rate.

The LT group included hospitalized cirrhotic patients with the same severity of liver disease as compared to LD group based on Child Pugh and MELD scores (Child Pugh: 11.0 ± 0.8 in LD *vs* 11.2 ± 1.2 in LT and MELD: 29.3 ± 2.1 in LD *vs* 29.8 ± 3.2 in LT, $P > 0.05$ for both comparisons). These patients also presented the same clinical and laboratorial features as the LD group and received the same diuretic treatment. The only difference between both groups is the fact that LT patients have been submitted to liver transplantation. Ten of the LT patients were using propranolol (40-80 mg/d) until the time of liver transplantation and their doses were also titrated to achieve a 20%-25% change in baseline heart rate.

Exclusion criteria

Co-morbidities such as diabetes, heart, pulmonary, autoimmune and neurological diseases automatically excluded subjects from the study. Patients receiving chronic treatment with angiotensin-converting enzyme inhibitors, angiotensin receptor blockers, renin inhibitors and corticosteroids were also excluded from the study. During liver transplantation, blood collection was suspended whenever the subject presented acute hemodynamic disarrangements and needed to use a vasoconstrictor.

Ethical aspects

The Ethics Committee of the Federal University of Minas Gerais approved the study. Informed consent was obtained from all included subjects. The research protocol did not interfere with any medical recommendations or prescriptions. Subject follow-up was guaranteed even in cases of refusal to participate in the study.

Study protocol

Protocol 1 - Evaluation of circulating RAS in outpatients using or not using propranolol: Blood

samples for PRA and angiotensin measurements were obtained from LD patients on a single occasion taking into account the inclusion and exclusion criteria for each group. Due to ethical reasons, no changes to the clinical approach were made for study purposes. Blood samples (10 mL) were collected through peripheral venipuncture in the morning after a fasting period of 8 h. All subjects rested in supine position for at least 30 min before blood sampling.

Protocol 2 - Evaluation of RAS and hemodynamic parameters during pre-anhepatic stage of liver transplantation in patients pre-treated with propranolol or not:

In the LT group, blood sampling was performed during the pre-anhepatic stage of liver transplantation and samples were obtained from the portal vein (10 mL) to evaluate RAS mediators. Hemodynamic parameters (cardiac output, cardiac index, systemic vascular resistance and systemic vascular resistance index) were determined simultaneously with the blood sampling. These measurements were obtained through invasive continuous monitoring *via* a Swan-Ganz catheter (CCOMBO/SvO₂, 110 cm/7.5 F, Edwards Lifesciences, Irvine, CA, USA), using Dixtal (DX 2020, Dixtal Biomedical, São Paulo, Brazil) and Vigilance (CEDV, Edwards Lifesciences, Irvine, CA, USA) monitors. Anesthesia for liver transplantation was induced by a rapid sequence of etomidate, fentanyl and succinylcholine and maintained by isoflurane (CAM~1.0) and atracurium until the blood sampling.

Blood collection: For all blood collections, samples were drawn into two sets of ice-cooled tubes-one containing 7.5% EDTA for PRA determinations and the other containing a cocktail of protease inhibitors for angiotensin measurements, as previously described^[14]. Blood samples were centrifuged at $\times 2000 g$ for 20 min at 4°C and plasma stored at -20°C^[14].

Plasma extraction and radioimmunoassays: Plasma samples were extracted using Bond-Elut cartridges (Analytichem International, Harbor City, CA), as described elsewhere^[15]. PRA as well as Ang I, Ang II and Ang-(1-7) concentrations were determined through radioimmunoassays, as detailed elsewhere^[15]. The recovery of ¹²⁵I-labeled Ang I, Ang II, and Ang-(1-7) was $79.2\% \pm 2.3\%$, $86.9\% \pm 0.8\%$ and $83.5\% \pm 0.9\%$, respectively. Results were expressed as nanograms of Ang I generated per mL of plasma per hour (ng Ang I /mL per hour) for PRA and pg/mL of plasma for Ang measurements.

Statistical analysis

Gaussian distribution of variables was evaluated by the Shapiro normality test. Results were reported as mean \pm SE or median, when appropriate. Unpaired *t* test was used for the comparison of means between groups. Mann-Whitney was used to compare non-parametric

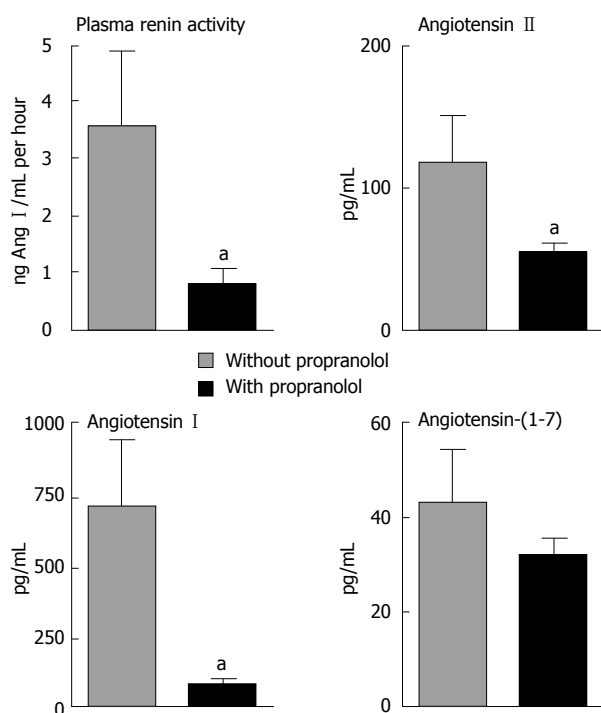


Figure 1 Peripheral circulating RAS profile in advanced liver disease outpatients (LD) receiving propranolol or not. Data are expressed as means \pm SE. ^a $P < 0.05$ for the comparison of LD with propranolol and LD without propranolol (unpaired t test).

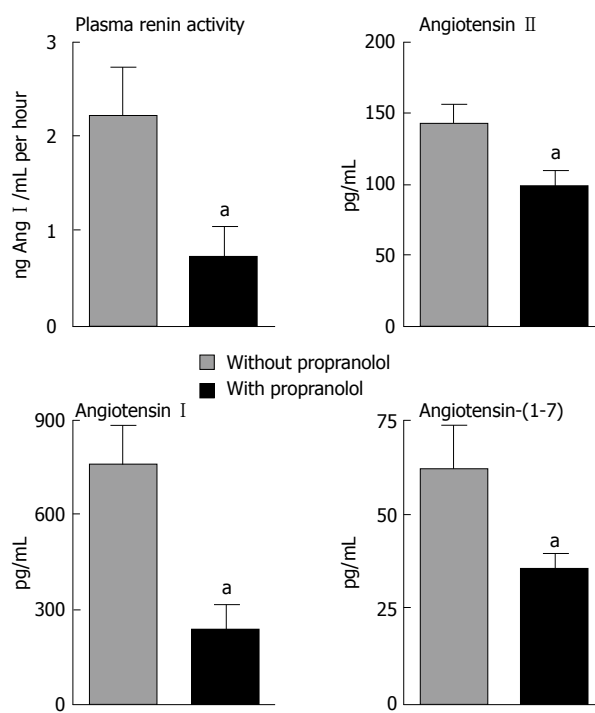


Figure 2 RAS profile of the splanchnic circulation in patients undergoing liver transplantation (LT), pre-treated with propranolol or not. Data are expressed as means \pm SE. ^a $P < 0.05$ for the comparison of LT with propranolol and LT without propranolol (unpaired t test).

data. The level of significance was set at $P < 0.05$. Graphpad PRISM was used for the statistical analyses.

RESULTS

Subject characteristics and casual measurements

The outpatients with liver cirrhosis (LD, $n = 16$) consisted of 9 males and 7 females from 44 to 66 years. The etiologies of liver failure in these subjects were: alcoholism in 4, bile cirrhosis in 1, virus C in 5, virus B in 1 and idiopathic causes in 5 patients. Clinical and laboratorial data revealed high Child Pugh and MELD scores that were very similar between the groups of patients using or not using propranolol (Table 1). Only serum sodium levels were significantly reduced in LD patients not receiving propranolol as compared to those treated with propranolol (Table 1).

Cirrhotic patients during liver transplantation (LT, $n = 21$) comprised 10 males and 11 females from 36 to 67 years. The etiologies for primary hepatic diseases in this group included virus C in 9, alcoholism in 4, bile cirrhosis in 2, virus B in 1 and idiopathic in 5 patients. The LT group without propranolol revealed reduced serum sodium levels compared to the LT group receiving propranolol. Other demographic characteristics and liver function scores were similar in both subgroups (Table 2).

Peripheral and splanchnic circulating RAS profile in LD and LT patients pre-treated with propranolol or not

As displayed in Figure 1, PRA and angiotensin I (Ang I) were lower in peripheral circulation of the LD group treated with propranolol in comparison to LD group not receiving propranolol (PRA: 3.54 ± 1.35 ng Ang I/mL

per hour *vs* 0.77 ± 0.28 ng Ang I/mL per hour; Ang I: 717.2 ± 39.0 pg/mL *vs* 79.8 ± 24.8 pg/mL, $P < 0.05$ for both comparisons). As shown in Figure 2, the same profile was observed for the LT group pre-treated with propranolol, which also presented a significant reduction of the PRA and Ang I plasma levels in the portal vein when compared to the LT group that was not treated with propranolol (PRA: 2.20 ± 0.51 ng Ang I/mL per hour *vs* 0.74 ± 0.30 ng Ang I/mL per hour; Ang I: 764.2 ± 117.0 pg/mL *vs* 235.0 ± 75.6 pg/mL, $P < 0.05$ for both comparisons).

LD patients receiving propranolol also exhibited a significant reduction in the levels of Ang II in peripheral circulation when compared to LD patients not using propranolol (Ang II: 117.2 ± 33.5 pg/mL *vs* 53.9 ± 6.5 pg/mL, $P < 0.05$, Figure 1). The same reduction of Ang II levels was observed in splanchnic circulation (portal vein) of the LT group under β -blockade in comparison to LT group not treated with propranolol (Ang II: 143.4 ± 13.3 pg/mL *vs* 96.9 ± 12.6 pg/mL, $P < 0.05$, Figure 2). Plasma levels of Ang-(1-7) were also reduced in splanchnic circulation of the LT group that was previously treated with propranolol in comparison to non-treated LT group (62.3 ± 10.9 pg/mL *vs* 35.5 ± 3.8 pg/mL, $P < 0.05$, Figure 2), whereas plasma Ang-(1-7) in peripheral circulation of the LD group did not differ significantly despite treatment or not with propranolol (Figure 1).

Ratios between Ang-(1-7) and Ang I levels, between Ang II and Ang I, and between Ang-(1-7) and Ang II in LD and LT groups are displayed in Tables 3 and 4, respectively. The ratio between Ang-(1-7) and Ang I indirectly reflects ACE2 activity, whereas the

Table 3 Ratios between angiotensins in peripheral circulation of advanced liver disease outpatients (LD) treated with propranolol or not

Angiotensin ratios	LD with propranolol (n = 9)	LD without propranolol (n = 7)
Ang II / Ang I	1.24 ± 0.30	0.21 ± 0.05 ^a
Ang-(1-7)/ Ang I	0.74 ± 0.19	0.09 ± 0.03 ^a
Ang-(1-7)/ Ang II	0.62 ± 0.06	0.42 ± 0.09

Data are expressed as mean ± SE. ^aP < 0.05 for the comparison of NLD with propranolol and NLD without propranolol (unpaired *t* test).

Table 4 Ratios between angiotensins in splanchnic circulation of patients undergoing liver transplantation (LT) pre-treated with propranolol or not

Angiotensin ratios	LT with propranolol (n = 10)	LT without propranolol (n = 11)
Ang II / Ang I	0.46 (0.26-2.64)	0.19 (0.13-0.24) ^a
Ang-(1-7)/ Ang I	0.27 (0.06-0.91)	0.05 (0.04-0.11)
Ang-(1-7)/ Ang II	0.43 ± 0.08	0.52 ± 0.12

Data are expressed as mean ± SE or median (25 and 75 percentile). ^aP < 0.05 for the comparison of LT with propranolol and LT without propranolol (unpaired *t* test for mean comparisons and Mann-Whitney test for median comparisons).

ratio between Ang II and Ang I indirectly estimates ACE activity. Both ratios were significantly increased in peripheral circulation of the LD group using propranolol in comparison to LD patients not receiving the β -blocker ($P < 0.01$, Table 3). Only Ang II / Ang I ratio was increased in splanchnic circulation of the LT group pre-treated with propranolol in comparison to LT patients that had not received propranolol ($P < 0.01$, Table 4), whereas Ang-(1-7)/Ang I did not significantly differ in splanchnic circulation of LT patients despite the previous treatment or not with propranolol. More importantly, the ratio between Ang-(1-7) and Ang II, which could represent the final functional relationship between RAS mediators, did not differ in either peripheral circulation of the LD group or in splanchnic circulation of the LT group, independently of the previous use or not of the β -blocker.

Hemodynamic parameters during pre-anhepatic stage of liver transplantation in LT pre-treated with propranolol or not

In order to demonstrate that our LT patients pre-treated with propranolol were adequately β blocked, we measured hemodynamic parameters during pre-anhepatic stage of liver transplantation. Accordingly, in LT patients pre-treated with propranolol, the cardiac output (8.9 ± 0.9 L/min *vs* 5.6 ± 0.6 L/min) and cardiac index (4.7 ± 0.4 L/min per m² *vs* 3.2 ± 0.3 L/min per m²) were reduced and the systemic vascular resistance ($604.2 \pm 65.0 \times 877.5 \pm 106.1$ dyn.s/cm⁵) and its index [$(1036 \pm 86) \times (1399 \pm 147)$ dyn.s/cm⁵ per m²] were increased in comparison to patients that had not previously received propranolol ($P < 0.05$ for all comparisons, Figure 3).

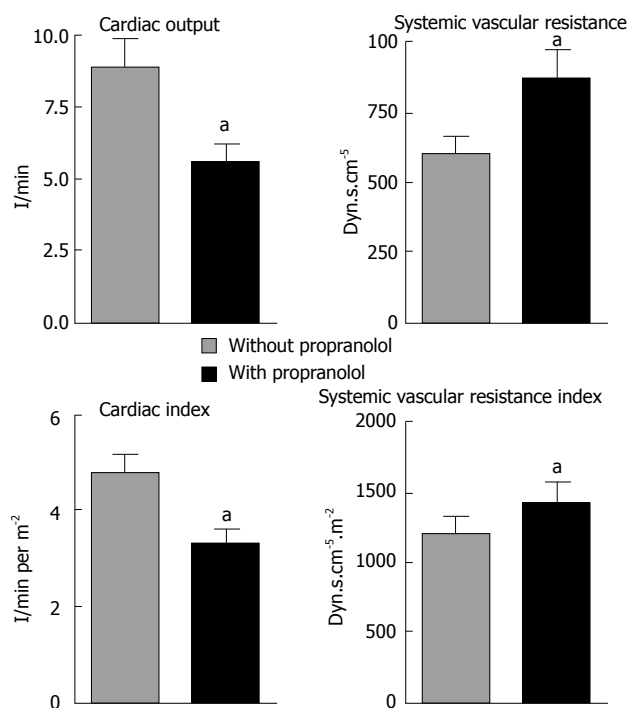


Figure 3 Hemodynamic parameters from cirrhotic patients during liver transplantation (LT) pre-treated with propranolol or not. Data are expressed as means ± SE. ^aP < 0.05 for the comparison of LT with propranolol and LT without propranolol (unpaired *t* test).

DISCUSSION

In general, our data showed that chronic treatment with propranolol in cirrhotic patients is characterized by marked changes in the precursors of RAS cascade (Renin and Ang I) with repercussion in RAS two main arms at the splanchnic and peripheral circulation. On the other hand, no changes were detected in the ratio between the two main RAS mediators [Ang-(1-7)/Ang II], which has been used to evaluate the final functional effect of the RAS. In parallel, the chronic use of propranolol produced hemodynamic changes, which were probably able to control the hyperdynamic circulation of cirrhotic patients. Taken together, these findings suggest that the reduction of hyperdynamic circulation produced by chronic treatment with propranolol in cirrhotic patients was associated with an overall RAS inhibition, but it was not due to changes in the balance between the two RAS arms: ACE-Ang-AT1 (vasoconstrictor) *versus* ACE2-Ang-(1-7)-Mas (vasodilator).

In splanchnic and peripheral circulation, the β -blockade in cirrhotic patients was characterized by reduced PRA and Ang I levels. These RAS components can lead to the synthesis of both Ang II and Ang-(1-7)^[5,16]. The cirrhotic patients receiving propranolol have reduced Ang II levels in the splanchnic and in the peripheral circulation as well as reduced Ang-(1-7) levels in the splanchnic circulation. In this regard, Blumenfeld *et al*^[12] previously suggested that β -blockade reduced Ang II levels and PRA in normotensive and hypertensive subjects by inhibiting prorenin processing to renin.

Ratios between angiotensins, especially the relationship between Ang-(1-7) and Ang II, have been used to estimate the final functional RAS effect^[2,3,4]. In this study, propranolol use was not able to change the ratio between Ang-(1-7) and Ang II in splanchnic and peripheral circulation of non-compensated cirrhotic patients, although there was an absolute decrease in both angiotensins. In parallel, systemic vascular resistance (SVR) and its index increased and cardiac output (CO) and its index decreased in non-compensated cirrhotic patients treated with propranolol. Similar hemodynamic changes in cirrhotic patients receiving propranolol have already been reported^[17-19] and attributed to β -adrenergic blockade^[20]. Since the relationship between Ang-(1-7) and Ang II remained unchanged in splanchnic and peripheral circulation of our cirrhotic patients, we could hypothesize that propranolol was not able to interfere with the final functional RAS effect upon vascular tone. Our data also suggest that the activity of the two main RAS enzymes, ACE and ACE2, were probably not reduced by propranolol use in cirrhotic patients, since the ratios between Ang II and Ang I and between Ang-(1-7) and Ang I were increased in peripheral circulation and the ratio between Ang II and Ang I was also elevated in splanchnic circulation. However, we can not exclude the possibility that other factors such as changes in the catabolism of Ang II or Ang-(1-7) could have contributed to the reduction in absolute levels of each peptide.

In cirrhotic patients, arteriolar vasodilation and diuretic administration cause a decreased effective arterial blood volume that stimulates vasopressor systems leading to high levels of PRA, circulating norepinephrine, and vasopressin^[21-23]. In this context, propranolol inhibits renin secretion and reduces vasodilation (SVR increase) in cirrhosis^[17], leading to a reduction of the relative arterial hypovolemia. These actions could oppose the activated vasopressor systems (RAS, sympathetic nervous system and vasopressin) and may be involved in the amelioration of the hyperdynamic circulation observed in our cirrhotic patients.

It should also be pointed out that we are aware of the limitations of our study design. For example, peripheral blood samples generally represent the cumulative expression of RAS in multiple tissues and may not reliably reflect molecular activity in the splanchnic circulation. For this reason, we did manage to collect samples from the portal vein during liver transplantation. However, it is still difficult to compare these findings to the samples collected in peripheral blood from outpatients. Nevertheless, some aspects of this study may increase the strength of our findings, such as the utilization of strictly defined inclusion and exclusion criteria and the well-established protocol for the measurements of PRA and angiotensins^[15].

In conclusion, results obtained with propranolol treatment in cirrhotic patients have been controversial^[20,24]. While in advanced liver disease with significant reduction of the hepatic venous pressure gradient propranolol treatment decreased the risk of ascites, spontaneous

bacterial peritonitis, hepatorenal syndrome and death^[24], in unselected cirrhotic patients the same β -blocker was not able to prevent varices and was associated with an increased number of adverse events^[20]. We believe that the use of propranolol in cirrhosis could change the prognosis of patients with hyperdynamic circulation and relative hypovolemia, but it is probably not able to interfere with potentially reversible liver fibrosis. Indeed, the use of propranolol did not alter the balance between the activity of the anti-fibrotic arm of the RAS, ACE2-Ang-(1-7)-Mas^[8], and of the pro-fibrotic arm, ACE-Ang II-AT1^[6]. For this purpose, many studies have suggested that ACE inhibitors and AT1 receptor blockers seemed to be effective^[25-29]. Their mechanisms of action probably involve not only the inhibition of Ang II formation or action but also the augmentation of Ang-(1-7) levels or effects^[5,29]. On the other hand, it should be mentioned that, mostly in advanced stages of cirrhosis, the ACE-Ang II-AT1 arm contributes to the maintenance of basal vascular tonus^[7] and therefore the use of AT1 receptor blockers or ACE inhibitors as antifibrotic therapies could not be well tolerated. Since propranolol administration seems to improve only the extrahepatic complications of the advanced cirrhotic patients, a possible therapeutic approach for human cirrhosis at this stage could be the combination of AT1 receptor blockers or ACE inhibitors with propranolol. Future studies with more powerful designs are obviously necessary to evaluate whether the use of propranolol at this stage of cirrhosis would enable the administration of AT1 receptor blockers or ACE inhibitors or even receptor Mas agonists to reduce liver fibrosis.

COMMENTS

Background

Recent studies have suggested that the Renin Angiotensin System (RAS) seems to be involved in cirrhosis. Non-selective β -adrenergic blockers have been widely used in treatment of portal hypertension in cirrhosis. However, the effect of propranolol on RAS mediators has still not been quantified. Since non-specific β blockade has been a standard approach to controlling the symptoms of portal hypertension and because the RAS seems to influence the outcome of portal hypertension and cirrhosis, it is reasonable to ask if there is a functional relationship between the RAS and beta-receptor system.

Research frontiers

This study represents an initial approach in understanding how non-specific β blockade affects RAS in cirrhotic patients by comparing the levels of plasma renin activity, Angiotensin (Ang) I, Ang II and Ang-(1-7), measured in the splanchnic and peripheral circulations of cirrhotic patients receiving or not receiving propranolol and by evaluating the effect of previous administration of propranolol on hemodynamic parameters during liver transplantation.

Innovations and breakthroughs

Chronic treatment with propranolol in cirrhotic patients is characterized by marked changes in the precursors of RAS cascade (Renin and Ang I) with repercussion in RAS two main arms in the splanchnic and peripheral circulation. On the other hand, no changes were detected in the ratio between the two main RAS mediators [Ang-(1-7)/Ang II]. Additionally, the treatment with propranolol seemed to be able to control the hyperdynamic circulation of cirrhotic patients probably due to an overall RAS inhibition, but without changes in the balance between the two RAS arms: ACE-Ang-AT1 (vasoconstrictor) versus ACE2-Ang-(1-7)-Mas (vasodilator).

Applications

Our data suggest that a possible therapeutic approach for advanced human cirrhosis could be the combination of AT1 receptor blockers or ACE inhibitors

with propranolol. Future studies with more powerful designs are obviously necessary to evaluate whether the use of propranolol at this stage of cirrhosis would enable the administration of AT1 receptor blockers or ACE inhibitors or even receptor Mas agonists to reduce liver fibrosis.

Peer review

In the current study, the investigators have taken the first steps to understand how $\beta 1$ and $\beta 2$ blockade affects RAS in patients. This is important because non-specific β blockade has been a standard approach to controlling the symptoms of portal hypertension.

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Pre-operative predictive factors for gallbladder cholesterol polyps using conventional diagnostic imaging

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Consequently, the discrepancy between those two scanning measurements was greater than for the non-cholesterol polyp group.

CONCLUSION: The clinical signs indicative of a cholesterol polyp include: (1) a polyp observed by US but not observable by CT scanning, (2) a smaller diameter on the CT scan compared to US, and (3) a discrepancy in its maximum diameter between US and CT measurements. In addition, US and the CT scan had low accuracy in predicting the polyp diameter compared to that determined by postoperative pathology.

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Key words: Cholesterol; Polyps; Gallbladder; Computed tomography; Ultrasonography

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Abstract

AIM: To determine the clinical data that might be useful for differentiating benign from malignant gallbladder (GB) polyps by comparing radiological methods, including abdominal ultrasonography (US) and computed tomography (CT) scanning, with postoperative pathology findings.

METHODS: Fifty-nine patients underwent laparoscopic cholecystectomy for a GB polyp of around 10 mm. They were divided into two groups, one with cholesterol polyps and the other with non-cholesterol polyps. Clinical features such as gender, age, symptoms, size and number of polyps, the presence of a GB stone, the radiologically measured maximum diameter of the polyp by US and CT scanning, and the measurements of diameter from postoperative pathology were recorded for comparative analysis.

RESULTS: Fifteen of the 41 cases with cholesterol polyps (36.6%) were detected with US but not CT scanning, whereas all 18 non-cholesterol polyps were observed using both methods. In the cholesterol polyp group, the maximum measured diameter of the polyp was smaller by CT scan than by US.

INTRODUCTION

The development of radiological diagnostic tools such as ultrasonography (US) and computed tomography (CT) scanning has led to an increased frequency of the diagnosis of gallbladder (GB) lesions, such as GB polyps^[1-3]. Because of the poor prognosis of GB malignancies, it is very important to distinguish between benign and malignant GB polyps so that malignant disease can be treated as soon as possible. Currently, clinical data such as the size and number of GB polyps and the age of the patient are used to help distinguish benign from malignant disease. Improved diagnostic methods are needed to differentiate between benign and malignant disease, and to determine which GB polyps

require surgical intervention^[4-6].

Therefore, we evaluated clinical data to determine which factors would help distinguish benign from malignant GB polyps. We retrospectively analyzed the preoperative US and CT findings in patients with GB polyps and compared the results with their postoperative gross and microscopic findings.

MATERIALS AND METHODS

Fifty-nine patients who underwent laparoscopic cholecystectomy for a GB polyp of around 10 mm between January 2006 and August 2007 were enrolled in this study. We divided these patients into two groups, a cholesterol polyp group and a non-cholesterol polyp group. Data were collected for clinical features such as gender, age, symptoms, size and number of polyps, presence of a GB stone, radiological data from the preoperative US and CT scanning, and postoperative pathology data.

We compared the radiologically measured maximum diameters of the GB polyps obtained by one radiologist with the postoperatively obtained pathologic measurements of maximum diameters obtained by one pathologist. Results are reported as the mean \pm standard deviation. For statistical analysis, a Chi-square, *t*-test and Fisher's Exact Test were used (SPSS version 15.0 software). A *P*-value < 0.05 was considered statistically significant.

RESULTS

Pathologic findings of the GB polyps

Of the 59 cases, 46 (78%) were pseudo-polyps such as a cholesterol polyp, inflammatory or hyperplastic polyp. Of these 46 pseudo-polyps, 41 (69.5%) were cholesterol polyps. True polyps were observed in 13 cases. Among the true polyps, 10 cases (17%) were adenomatous polyps and three cases (5.0%) were malignant.

Clinical findings of the GB polyps

Of the 59 patients, 37 patients were male and 22 were female. No difference was observed in gender ratios for the cholesterol polyp group (M:F = 25:16) and the non-cholesterol polyp group (M:F = 12:6 *P* > 0.05). The mean ages for each group were 40.98 ± 9.41 for the cholesterol polyp group and 48.39 ± 16.87 for the non-cholesterol polyp group. The former group had a significantly lower mean age (*P* = 0.044).

Five patients had presenting symptoms, which included three cases of indigestion, one case of right upper quadrant pain and discomfort and one case with fever suggesting cholecystitis. Three cases of cholesterol polyps (7.3%) and two cases of non-cholesterol polyps (11.1%) were associated with a GB stone. The factors associated with the metabolic syndrome were analyzed in the two groups. The mean body mass index (BMI) was 24.83 ± 2.92 kg/m² in the cholesterol polyp group and 23.80 ± 3.23 in the non-cholesterol polyp group; the mean homeostasis model assessment of insulin

Table 1 Clinical and laboratory characteristics of patients with gallbladder polyps (mean \pm SD)

Histologic finding	Cholesterol polyp	Non cholesterol polyp	P-value
Age (yr)	40.98 \pm 9.41	48.39 \pm 16.87	< 0.05
Sex (male/female)	25/16	12/6	0.677
Height (m)	1.6703 \pm 0.09	1.65 \pm 0.07	0.595
Weight (kg)	69.39 \pm 13.26	64.85 \pm 10.73	0.207
Cholelithiasis(case)	3	2	0.63
BMI (kg/m ²)	24.83 \pm 2.92	23.80 \pm 3.23	0.245
Fasting glucose (mg/dL)	97.05 \pm 21.76	96.94 \pm 15.0	0.985
Insulin (μ U/mL)	10.54 \pm 3.63	9.47 \pm 2.71	0.412
Homa-IR	1.49 \pm 1.85	1.26 \pm 1.35	0.64
HbA1c (%)	5.58 \pm 0.74	5.41 \pm 0.23	0.553
US size (mm)	9.95 \pm 2.31	11.94 \pm 4.02	< 0.05
CT size (mm)	6.77 \pm 2.65	9.78 \pm 5.19	< 0.05
Pathology size (mm)	4.83 \pm 2.97	11.06 \pm 5.11	< 0.01

Table 2 The number of polyps in cases with cholesterol and non-cholesterol polyps *n*(%)

	Cholesterol polyp	Non-cholesterol polyp	Total
Ultrasonographic findings (<i>P</i> < 0.01)			
Polyp number	19 (46.3)	16 (88.8)	35 (59.3)
Multiple	22 (53.7)	2 (11.1)	24 (40.7)
Total	41 (69.5)	18 (30.5)	59 (100.0)
Pathologic finding (<i>P</i> < 0.01)			
Polyp number	11 (26.8)	15 (83.3)	26 (44.1)
Multiple	30 (73.2)	3 (16.7)	33 (55.9)
Total	41 (69.5)	18 (30.5)	59 (100.0)

resistance (HOMA-IR) was 1.49 ± 1.85 and 1.26 ± 1.35 respectively, the mean HbA1c was 5.58 ± 0.74 (%) and 5.41 ± 0.23 , respectively. The mean values for all these factors were slightly higher in the cholesterol polyp group but they were not statistically significant (*P* > 0.05) (Table 1).

The number of GB polyps

In US, a single GB polyp was observed in 35 cases (59.3%) and multiple GB polyps were observed in 24 cases (40.7%). The proportion of multiple polyps in the cholesterol polyp group was 53.7% (22 out of 41 cases), which was higher than in the non-cholesterol polyp group (11.1%: 2 out of 18 cases, *P* = 0.002). For the postoperative pathology examinations, these proportions increased; 73.2% (30 out of 41 cases) and 16.7% (3 out of 18 cases), respectively (*P* < 0.001) (Table 2).

The discrepancy in maximum diameter between US and CT scanning

The preoperative mean maximum diameters measured by US in the cholesterol polyp group and the non-cholesterol polyp group were 9.95 ± 2.31 mm and 11.94 ± 4.02 mm, respectively, whereas for the CT scan they were 6.77 ± 2.65 mm and 9.78 ± 5.19 mm, respectively. The mean values for CT scanning tended to be smaller than for US.

The discrepancies in maximum diameters between US and CT scanning were 5.66 ± 3.87 mm in the

Table 3 The difference in the maximum polyp size between cholesterol and non-cholesterol polyps (mean \pm SD)

	Cholesterol polyp	Non-cholesterol polyp	P-value
US-CT size difference (mm)	5.66 \pm 3.87	2.17 \pm 2.12	0
US size > CT size ¹	40/41	12/18	0.002
CT undetectable rate(%)	15/41 (36.6)	0/18 (0)	0.001
US-pathologic size difference (mm)	5.12 \pm 3.42	0.89 \pm 3.69	0

¹Indicates number of patients having a larger size with US than CT.

cholesterol polyp group and 2.17 \pm 2.12 mm in the non-cholesterol polyp group and this difference was statistically significant ($P < 0.01$). In 40 out of 41 cholesterol polyps (97.6%) and 12 out of 18 non-cholesterol polyps (66.6%) the diameters were smaller with CT scanning than with US ($P < 0.01$).

All 18 cases in the non-cholesterol polyp group were detected both by US and CT whereas 15 cases in the cholesterol polyp group among 41 (36.6%) were detected by US but not by CT scanning ($P < 0.01$, Table 3).

The discrepancy between preoperatively and postoperatively measured maximum polyp diameters

The pathologically measured mean maximum diameters were 4.83 \pm 2.97 mm in the cholesterol polyp group and 11.06 \pm 5.11 mm in the non-cholesterol polyp group ($P < 0.01$). When we compared these values with the preoperatively US measurements the discrepancies between preoperative and postoperative measurements were 5.12 \pm 3.42 mm in the cholesterol polyps and 0.89 \pm 3.69 mm in the non-cholesterol polyps ($P < 0.01$, Table 3).

The correlation between radiologically measured and pathologically measured polyp diameters

The non-cholesterol polyps showed statistically significant linear correlations between the actual maximum diameter from the pathology examination and the preoperative US measured diameter (correlation coefficient 0.698) and the CT measured diameter (correlation coefficient 0.746, $P < 0.01$). The cholesterol polyps, however, did not show this correlation ($P > 0.05$, Table 4).

DISCUSSION

The correct diagnosis of cholesterol polyps, which account for most of the pseudo-polyps of the GB, will help prevent unnecessary surgery and follow-up examinations. In this study, we attempted to characterize the features of the cholesterol polyp and determine accurate radiological predictive factors. Age is known to have a significant association with malignant polyps and is considered an independent risk factor^[5-7]. This study also found that patients with non-cholesterol polyps had a higher mean age than did the patients in the cholesterol polyp group. Metabolic syndrome is also known to have a close relationship with the development of cholesterol

Table 4 The correlation of size between cholesterol and non-cholesterol polyps

Correlation coefficients		Pathologic size	US size	CT size
Pathologic size	Non-cholesterol polyp	1	0.698 ^b	0.746 ^b
	Cholesterol polyp	1	0.181	0.324
US size	Non-cholesterol polyp	0.698 ^b	1	0.925 ^d
	Cholesterol polyp	0.181	1	0.427 ^c
CT size	Non-cholesterol polyp	0.746 ^b	0.925 ^d	1
	Cholesterol polyp	0.324	0.427 ^c	1

^b $P < 0.01$ vs Pathologic size, ^b $P < 0.05$ vs US size.

polyps^[2,8,9]. Although the patients with cholesterol polyps had higher levels of the BMI, HOMA-IR, and HbA1c, the differences did not reach statistical significance. The sample size might have been too small to detect any differences.

Regarding the number of polyps in the GB, it is also known that a single polyp is more likely to be a malignant polyp, which prompts the need for more aggressive interventions when a single polyp is identified compared to multiple polyps^[5,10]. We found a similar tendency among our study population. The patients with cholesterol polyps more frequently had multiple polyps than did the patients with non-cholesterol polyps. It is well known that the size of a GB polyp is related to malignancy. Many studies have reported that a GB polyp ≥ 10 mm has a high risk of being a malignancy and this size is one of the criteria for surgical intervention^[4,11-13]. However, we also have observed that a benign polyp, such as a cholesterol polyp, can be as large as 10 mm. Therefore, size may not afford an accurate distinction between benign and malignant polyps^[14,15].

In cases with a cholesterol polyp, we observed discrepancies in the size and number of polyps between the preoperative radiological measurements and the postoperative pathology measurements. The postoperative pathology of cholesterol polyps had a smaller size and higher multiplicity than did the preoperative radiological studies. A possible explanation for this finding is that the cholesterol polyp might be damaged during the laparoscopic cholecystectomy or during handling of the GB tissue considering its histological fragility and weakness. The cholesterol polyp had low correlation coefficients in the comparisons between the pathologically measured size after surgery and the radiologically measured sizes prior to surgery. Therefore, the radiological studies are limited in obtaining the correct measurements for cholesterol polyps.

In conclusion, the cholesterol polyp has a tendency to be observed more frequently in younger patients and has higher multiplicity. The predictive signs for a cholesterol polyp, a benign tumor, include: a polyp observable by US but not CT scanning, a discrepancy ≥ 5 mm in the maximum diameter of the polyp between the US and CT measurements, a smaller diameter of the polyp by CT compared to US, and a low correlation between the diameter of the polyp from postoperative pathology and

the preoperative radiological measurements.

We suggest that it would be more efficient to make a flexible and tailored follow up plan or treatment plan for GB polyps based on the above mentioned signs rather than fixed or inflexible guidelines. In addition, the preoperative radiological measurement of diameter is of predictive value for the postoperatively measured actual diameter only for non-cholesterol polyps. For cholesterol polyps, the preoperative radiological measurements are limited in their prediction of postoperative pathology diameter. Therefore, methods that are more accurate for the preoperative diagnosis of cholesterol polyps are needed.

COMMENTS

Background

The development of radiological diagnostic tools has led to an increased frequency of the diagnosis of gallbladder (GB) lesions, such as GB polyps.

Research frontiers

It is very important to distinguish between benign and malignant GB polyps because of the poor prognosis of GB malignancies. Improved diagnostic methods are needed to differentiate benign from malignant disease, and to determine which GB polyps require surgical intervention.

Innovation and breakthroughs

The predictive signs for a cholesterol polyp, the most common benign GB polyp, include: a polyp observable by ultrasonography (US) but not computed tomography (CT) scanning, a discrepancy ≥ 5 mm in the maximum diameter of the polyp between US and CT measurements, a smaller diameter of the polyp by CT than by US.

Applications

This study should help to distinguish a cholesterol polyp from a non-cholesterol polyp. It would be more efficient to make a flexible and tailored follow up plan or treatment plan for GB polyps.

Peer review

The concept of this study is useful.

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Micronucleus analysis in patients with colorectal adenocarcinoma and colorectal polyps

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INTRODUCTION

Genomic instability plays an essential role in the development and progression of human colorectal cancer (CRC)^[1]. Two major types of genetic instability have been described in CRC: chromosomal instability and microsatellite instability^[1]. About 60% of CRCs develop through the chromosomal instability pathway, which is characterized by losses and gains of chromosomes (aneuploidy), as well as losses of heterozygosity^[1].

CRC progresses through four distinct clinical stages that are described as dysplastic crypts, small benign tumors, malignant tumors invading surrounding tissues, and finally metastatic cancer. This progression involves several genetic changes such as inactivation of tumor suppressor genes and activation of oncogenes^[2]. Mutations of the adenomatous polyposis coli (*APC*) gene are considered the earliest^[3] and most prevalent genetic changes in colorectal tumorigenesis. More than 85% of colon cancers are estimated to have a somatic mutation of *APC*^[4]. Furthermore, a large number of genes that trigger chromosomal instability have been identified in yeast in the past^[5]. The underlying mechanisms leading to chromosomal instability in colorectal cancer remain to be characterized. The DNA double-strand break (DSB) is regarded as the most critical of all DNA lesions^[6,7], and it has been shown that defects in the cellular response to DSBs can lead to genetic alteration, chromosomal instability, and ultimately malignant transformation^[8].

The genome damage to the lymphocytes of peripheral blood has been widely used as a biomarker of genotoxic environmental factors, and long-term studies have demonstrated its validity and high clinical productivity^[9]. Micronucleus (MN) is an acentric chromosome fragment or whole chromosome that is left behind during mitotic cellular division and appears in the cytoplasm of interphasic cells as a

Abstract

AIM: To determine, by counting micronucleus (MN) frequencies, whether chromosomal or DNA damage have an effect on the pathogenesis of early colorectal adenocarcinoma (CRC).

METHODS: We analyzed MN frequencies in 21 patients with CRC, 24 patients with colon polyps [10 neoplastic polyps (NP) and 14 non-neoplastic polyps (NNP)] and 20 normal controls.

RESULTS: MN frequency was significantly increased in CRC patients and in NP patients compared with controls (3.72 ± 1.34 , 3.58 ± 1.21 vs 1.97 ± 0.81 , $P < 0.001$). However, there was no difference in the MN frequency between CRC patients and NP patients ($P > 0.05$). Similarly, there was no difference in the MN frequency between NNP patients (2.06 ± 0.85) and controls ($P > 0.05$).

CONCLUSION: Our results suggest increased chromosome/DNA instabilities may be associated with the pathogenesis of early CRC.

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Key words: Colorectal adenocarcinoma; Colon polyp; Micronucleus; Genetic instability

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small additional nucleus^[10]. The formation of MN in dividing cells is the result of chromosome breakage due to unrepaired or mis-repaired DNA lesions, or chromosome malsegregation due to mitotic malfunction. These events may be induced by oxidative stress, exposure to clastogens or aneugens, genetic defects in cell cycle checkpoint and/or DNA repair genes, as well as deficiencies in nutrients required as co-factors in DNA metabolism and chromosome segregation machinery^[11-14]. All these events can cause the formation of MN through chromosomal rearrangements, altered gene expression or aneuploidy, effects associated with the chromosome instability phenotype often seen in cancer^[15,16].

The MN frequency test, widely accepted for *in vitro* and *in vivo* genotoxicity investigations, is a sensitive marker of genomic damage^[17,18]. The presence of an association between MN induction and cancer development is supported by a number of observations. The most substantiated include: the high frequency of MN in untreated cancer patients and in subjects affected by cancer-prone congenital diseases, e.g. Bloom syndrome or ataxia telangiectasia^[15,19], the presence of elevated MN frequencies in oral mucosa, used as a surrogate biomarker of cancer in clinical chemoprevention trials^[20], the correlation existing between genotoxic MN-inducing agents and carcinogenicity, e.g. ionizing and ultraviolet radiation^[21,22].

A major question in cancer genetics is to what extent chromosome or genetic instability is an early event and thus a driving force of tumorigenesis^[23,24]. The aim of this study was to determine, by counting MN frequencies, whether chromosomal or DNA damage has an effect on the pathogenesis of early CRC.

MATERIALS AND METHODS

Patients

This study was conducted between May 2008 and September 2008 in the Erzurum Training and Research Hospital. Twenty-one patients with colorectal adenocarcinoma and 24 patients with colorectal polyps were studied. The study was conducted using colonoscopic specimens from subjects with the established diagnosis of colorectal polyps or colorectal adenocarcinoma in histologic analysis. Specimens were separated for each level and placed in 10% formalin solution. The pathologic specimens were reviewed independently by two pathologists.

Pathologists were blinded to the subject's clinical history, the colonoscopic findings, and the results of the Hematoxylin-Eosin staining assay. Pathologic reading was determined for each biopsy slide with an overall pathologic diagnosis determined for each subject.

We performed MN analysis in 21 (12 females and 9 males; mean age: 57.62 ± 10.84 years) patients with CRC, in 10 (4 females and 6 males; mean age: 52.44 ± 8.36 years) patients with NP, in 14 (6 females, 8 males; mean age: 52.92 ± 9.14 years) patients with NNP and in 20 (8 females and 12 males; mean age: 50.25 ± 9.38 years) healthy controls. The patients were selected

from non-smoking and nonalcoholic subjects. None of the subjects had a history of viral infection, bacterial infection or any metabolic diseases. The patients had not been treated with chemotherapy or radiotherapy during the last 4 mo. The patient and control groups were chosen for their similar habits. The hospital Ethical Committee approved the human study. All patients were analyzed prior to treatment.

Micronucleus analysis

For MN analysis, 2 mL of heparinized blood was drawn from each individual. Lymphocyte cultures were established by adding 0.5 mL of whole blood to 5 mL karyotyping medium (Biological Industries, Beit Haemek, Israel) with 2% phytohemagglutinin M (PHA; Biological Industries) according to standard techniques. The culture was kept at 37°C for 72 h. Cytochalasin B (6 µg/mL, Sigma, USA) was added after 44 h of culture to block cytokinesis, allowing the identification of lymphocytes dividing in culture. Cells that had undergone the first mitosis were thus recognized as binucleated cells and were selectively screened for the presence of MN. The cells were then treated hypotonically with 0.075 mol/L KCl for 5 min at room temperature, and fixed in methanol/acetic acid (3:1). Cells were dropped onto slides and stained with 5% Giemsa in phosphate buffer (pH 6.8) for 5 min. A thousand binucleated cells from each case were examined for MN by an experienced observer^[25].

Statistical analysis

The MN rates were analyzed statistically by student's *t*-test. To evaluate the correlations between the age, sex, and MN rates, the coefficients of Spearman ρ correlation were calculated. A *P* value less than 0.05 was considered to be significant.

RESULTS

MN frequencies and clinical data obtained from the patient and control groups are shown in Table 1. According to these results, the mean MN frequency was significantly increased in CRC patients compared with controls (3.72 ± 1.34 vs 1.97 ± 0.81 , $P < 0.001$). Similarly, the mean MN frequency was significantly increased in NP patients compared with controls (3.58 ± 1.21 vs 1.97 ± 0.81 , $P < 0.001$). However, there was no difference in the mean MN frequency between CRC patients, and NP patients ($P > 0.05$). Similarly, there was no difference in mean MN frequency between NNP patients and controls (2.06 ± 0.85 vs 1.97 ± 0.81 , $P > 0.05$). On the other hand, the mean MN frequencies did not correlate with patients' age or sex in the CRC patients (for each, $P > 0.05$). Similarly, the mean MN frequencies did not correlate with patients' age or sex in the colon polyp patients (for each, $P > 0.05$).

DISCUSSION

CRCs progress through a series of clinical and his-

Table 1 Micronucleus (MN) results of the patients with colorectal cancer and colon polyps and healthy controls (mean \pm SE)

	Sex (F/M)	Age (yr)	Age at diagnosis (yr)	MN/1000 BN
CRC patients (<i>n</i> = 21)	12/9	57.62 \pm 10.84	56.98 \pm 9.45	3.72 \pm 1.34
NP patients (<i>n</i> = 10)	4/6	52.44 \pm 8.36	51.68 \pm 8.54	3.58 \pm 1.21
NNP patients (<i>n</i> = 14)	6/8	52.92 \pm 9.14	50.48 \pm 8.29	2.06 \pm 0.85
Controls (<i>n</i> = 20)	8/12	50.25 \pm 9.38		1.97 \pm 0.81

CRC: Colorectal adenocarcinoma; NP: Neoplastic polyp; NNP: Non-neoplastic polyp.

topathological stages ranging from dysplastic crypts through small benign tumors to malignant cancers. This progression is the result of a series of genetic changes that involve activation of oncogenes and inactivations of tumor suppressor genes^[26]. In colorectal cancer, chromosomal instability is the major form of genetic instability^[27]. It is generally agreed that colorectal cancers develop as a consequence of accumulation of mutations in key genes such as *K-Ras*, *ApC*, and *p53* that are critical for regulating cell proliferation or cell cycle checkpoint control. In humans, the development from early adenomas to metastatic carcinomas takes somewhere from 20 to 40 years; it is believed that genetic instability plays a key role in accelerating the rate of mutation in cancerous cells^[28].

CRCs exhibit a defect in chromosome segregation, leading to frequent gains or losses of chromosomes ($> 10^2$ per chromosome per generation)^[28]. Chromosome instability has been detected in the smallest adenoma, suggesting that chromosome instability may occur at very early stages of colorectal carcinogenesis^[29]. Extensive research during the past has led to the identification of genes that play a major role in the development of colorectal cancer. For example, mutations or deletions of the adenomatous polyposis coli (*APC*) gene, encoding a 310-kDa cytoplasmic protein^[30,31], are commonly found in inherited familial adenomatous polyposis patients and in sporadic colorectal cancers^[32,33]. Such mutations appear to be an early event during colorectal tumorigenesis^[4].

The most commonly affected gene in sporadic colon cancer with defective DNA mismatch repair (MMR) is *hMLH1*, with the primary mechanism of gene inactivation being hypermethylation of the promoter^[34]. These tumors account for approximately 15% of sporadic colon cancers. The majority of sporadic colon cancers (85%), however, are proficient in DNA MMR but show another form of genomic instability at the gross chromosomal level, which has been called chromosomal instability. Such chromosomal instability represents the end result of a number of processes, including mutations in mitotic checkpoint genes, microtubule spindle defects, and telomere dysfunction^[35].

Two types of genetic instability have been identified, with chromosomal instability predominating^[1,36]. The molecular basis for chromosomal instability is just beginning to be explored^[37]. A large number of gene alterations can give rise to chromosomal instability in *Saccharomyces cerevisiae*^[5,38]. These genes include

those involved in chromosome condensation, sister-chromatid cohesion, kinetochore structure and function, and microtubule formation and dynamics as well as checkpoints that monitor the progress of the cell cycle. To date, the only genes implicated in aneuploidy in human tumor cells are those of the latter class. Heterozygous mutations in the mitotic spindle checkpoint gene *hBUB1* were detected in a small portion of colorectal tumors with the chromosomal instability^[39].

The identification of aneuploidy at early stages of tumor formation in *MYH*- and *APC*-mutant polyps is interesting also in view of previous reports showing that loss of *APC* function in primary mouse cell lines results in chromosomal instability due to a kinetochore attachment defect at mitosis^[36]. It is generally accepted that *APC*'s main tumor suppressing activity resides in its capacity to bind and regulate Wnt/ β -catenin signal transduction^[40]. However, additional *APC* functions in cytoskeletal organization, mitotic spindle assembly, cell migration, and apoptosis may play important roles in tumor progression and malignant transformation^[40,41].

It has been demonstrated that chromosomes display nonrandom changes in cancer cells. These include structural rearrangements, e.g. deletions, amplifications or translocations that arise from breaks in DNA, as well as alterations in the number of intact chromosomes, known as whole-chromosome missegregations, originating from errors in cell division (mitosis). As a result of the accumulation of such processes, chromosomal instability is thought to play a key role in tumor development^[40].

In the present study, we investigated whether cytogenetic abnormalities participate in the pathogenesis of early CRC. Cytogenetic endpoints are sensitive biomarkers that are widely accepted to evaluate chromosome damage^[42,43]. MN assay provides a measure of both chromosome breakage and chromosome loss or nondisjunction in clastogenic and aneugenic events, respectively^[11,13].

MN assay is a sensitive indicator of exogenously or endogenously caused genetic damage and MN frequency has become an important end point in genotoxicity testing both *in vivo* and *in vitro*^[17,18]. Elevated levels of MN are indicative of defects in DNA repair and chromosome segregation which could result in generation of daughter cells with altered gene dosage, or deregulation of gene expression that could lead to the evolution of the chromosome instability phenotype often seen in cancer^[10,11,15,21]. These considerations give

mechanistic support to a possible causal association between MN frequency and the risk of cancer. A recently published cohort study linking the frequency of micronuclei in lymphocytes of healthy subjects to the risk of cancer reported stomach cancer among the sites most specifically associated with micronuclei frequency^[44]. Similar findings have also been reported for preneoplastic lesions of colon^[45], esophagus^[46] and cervix^[47]. In particular, the higher risks noted for stomach and intestinal cancers, are in agreement with the literature, which emphasizes the role of chromosome rearrangements in the early stages of these tumours^[47,48].

Our study, which showed increased MN frequencies in the lymphocytes of CRC and colon polyp patients, could support these observations, as the induction of changes in DNA that lead to mutations plays a role in carcinogenicity. Establishment of inherited susceptibility factors is important in recognizing individuals at a higher risk of developing CRC, so that they may benefit from early detection and prevention programs. Many investigators have demonstrated genomic instability and abnormalities in patients with CRC^[49-51]. Further, experimental evidence shows that early colorectal adenomas have allelic imbalance^[52]. *bCDC4* mutations have been shown to occur early in colorectal tumorigenesis^[53].

An association between MN and cancer has been reported^[19]. The causes of this association may be structural chromosomal aberrations and aneuploidy^[19]. The presence of an association between the frequency of micronuclei in lymphocytes and cancer risk has been suggested^[13,44]. Our findings of a high level of MN frequency in patients with CRC or NP seem to support this association. Thus, MN assay may be performed in lymphocytes as an indicator of genomic instability relevant to colorectal tumorigenesis.

In conclusion, our results indicate that the increased MN frequency in lymphocytes of patients with CRC and NP may reflect genomic instability or deficiency of DNA repair capacity. Further, these results suggest increased chromosome/DNA instabilities may be associated with the pathogenesis of early CRC.

COMMENTS

Background

It is known there is an increased micronucleus (MN) frequency rate in neoplastic disease. Colorectal adenocarcinoma (CRC) is a common cause of cancer-related deaths worldwide, despite improved diagnostic and therapeutic implications. Hence, early diagnosis has critical importance. The aim of this study was to determine, by counting MN frequencies, whether chromosomal or DNA damage has an effect on the pathogenesis of early CRC.

Research frontiers

The MN frequency test, widely accepted for in vitro and in vivo genotoxicity investigations, is a sensitive marker of genomic damage. Therefore, in this study, we aimed to determine, by assessing MN rates, whether genetic impairment and DNA damage have an effect on the pathogenesis of CRC.

Innovations and breakthroughs

Our results suggest increased genomic instability may be associated with the pathogenesis of early CRC. The identification of increased MN frequency rate in patients with colorectal lesions may be helpful in the early diagnosis of CRC.

Applications

MN analysis has come into use as a sensitive means of monitoring DNA damage. MN analysis may be used as a marker to estimate the risk of CRC.

Terminology

Micronucleus (MN): MN is an acentric chromosome fragment or whole chromosome that is left behind during mitotic cellular division and appears in the cytoplasm of interphasic cells as a small additional nucleus.

Peer review

This study indicated genetic impairment and genetic instability may play an important role in CRC. Further, MN frequency is a promising biomarker for assessing the risk of neoplastic progression in colorectal adenocarcinoma.

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RAPID COMMUNICATION

Serum neopterin levels in children with hepatitis-B-related chronic liver disease and its relationship to disease severity

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Abstract

AIM: To evaluate serum neopterin levels and their correlations with liver function tests and histological grade in children with hepatitis-B-related chronic liver disease.

METHODS: The study population comprised 48 patients with chronic active hepatitis B, 32 patients with hepatitis-B-related active liver cirrhosis and 40 normal controls. Serum neopterin was measured using an enzyme-linked immunosorbent assay.

RESULTS: The mean \pm SD serum neopterin levels were 14.2 ± 5.6 nmol/L in patients with chronic hepatitis, 20.3 ± 7.9 nmol/L in patients with liver cirrhosis and 5.2 ± 1.4 nmol/L in control group. Serum neopterin levels were significantly higher in patients with chronic hepatitis ($P = 0.005$) and cirrhosis patients ($P = 0.008$), than in control subjects. Cirrhotic patients had significantly higher serum neopterin levels than patients with chronic hepatitis ($P = 0.004$). There was a positive correlation between serum neopterin levels and alanine aminotransferase levels in patients with chronic hepatitis ($r = 0.41$, $P = 0.004$) and cirrhotic patients ($r = 0.39$, $P = 0.005$). Positive correlations were detected between serum neopterin levels and inflammatory score in patients with chronic hepatitis ($r = 0.51$, $P = 0.003$) and cirrhotic patients ($r = 0.49$, $P = 0.001$).

CONCLUSION: Our results suggest that serum neopterin levels can be considered as a marker of inflammatory activity and severity of disease in children with hepatitis-B-related chronic liver disease.

INTRODUCTION

Neopterin is a pteridine derivative produced by macrophages activated under the control of gamma-interferon and released from T-cells by the activation of the cellular immune system^[1]. It has been demonstrated that there is a relation between neopterin levels in biological materials, the changes in their elimination rates and various pathological conditions. In addition to its association with activation of cell-mediated immunity and with cell expansion, significant changes were seen in neopterin levels and elimination rates in viral diseases (for example viral hepatitis)^[2,3], atypical phenylketonuria^[4], organ and tissue rejection^[5], autoimmune diseases (such as rheumatoid arthritis and systemic lupus erythematosus^[6]), genital cancer and hematologic neoplastic disorders^[7,8]. In all these cases, enhanced concentrations of neopterin have been shown to have prognostic significance^[9].

In chronic active hepatitis, necrosis is observed as disseminated to the parenchyma and the perlobular consisting of lymphocytes and plasma cells. The gamma-interferon released from T-lymphocytes in the area stimulates and activates macrophages^[10]. Lymphocytic cell infiltration was shown, in addition to the macrophages within the fibrous bands in the liver of patients with cirrhosis resulting from various etiologies^[11]. Thus, it is suggested that neopterin secreted from the inflammation-activated macrophages can be an indication of the inflammation in the liver in chronic

liver diseases^[10-12].

Studies in adult patients with acute hepatitis, chronic hepatitis and cirrhosis have shown that serum neopterin levels are elevated and this elevation is correlated with the severity of disease. However, there is no data about serum neopterin concentrations in children with chronic hepatitis B and liver cirrhosis. Therefore, we investigated serum neopterin concentrations in children with hepatitis-B-related chronic liver disease and correlated these concentrations with liver function tests and inflammatory activity of the liver. The aim of this study was to demonstrate a possible relationship between serum neopterin levels and severity of the disease.

MATERIALS AND METHODS

The study population comprised 48 patients with chronic active hepatitis B, 32 patients with hepatitis-B-related active liver cirrhosis and 40 normal controls. The control group consisted of otherwise healthy, age- and sex-matched children whose biochemical tests were also within normal limits. The study was performed according to the Declaration of Helsinki, and all parents gave informed consent for the participation of their children in the study.

Chronic active hepatitis B was diagnosed on the basis of Hepatitis B surface antigen (HbsAg) and Hepatitis B e antigen (HbeAg) positivity in serum for a period over 6 mo, positive HBV-DNA determined at least twice in one-month intervals (> 5 pg/mL), and evidence of chronic active hepatitis B in a liver biopsy carried out within the last 6 mo. None of the patients in this group had Hepatitis C or Hepatitis D infections, decompensated liver disorder, autoimmune hepatitis, α_1 -antitrypsin deficiency, Wilson disease or any other liver disease. The patients were evaluated before no treatment was initiated.

Hepatitis-B-related active liver cirrhosis was diagnosed by clinical, serological, and biochemical tests as well as histopathological investigation of liver biopsy. The cirrhotic patients were classified by the Child-Pugh classification defined by Pugh *et al*^[13].

Liver function tests (serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma-glutamyl transpeptidase (GGT), alkaline phosphatase (AP) and albumin) were also performed in all subjects using an autoanalyzer.

Serum neopterin levels were examined using an enzyme-linked immunosorbent assay (ELISA) kit (Neopterin ELISA, IBL Immuno-Biological-Laboratories, Hamburg).

All patients with chronic hepatitis and liver cirrhosis underwent liver biopsy. Liver biopsy was performed according to the Menghini technique. The biopsy material was kept in 10% formaldehyde solution and evaluated by a pathologist experienced in liver pathology. In the samples obtained from the patients with chronic hepatitis and liver cirrhosis, histological activity index (HAI) score was defined as suggested by Knodell *et al*^[14] and modified by Desmet *et al*^[15]. It was graded 0-18 by adding the scores for periportal \pm bridging necrosis

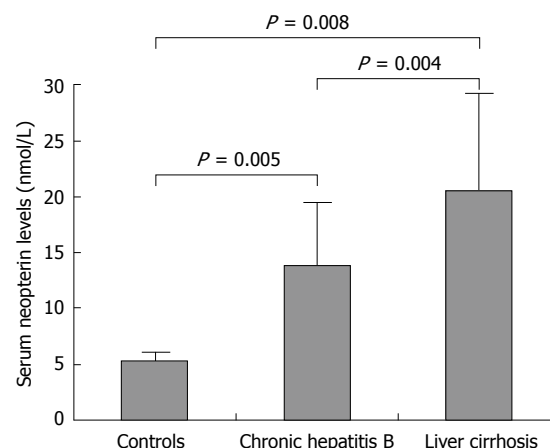


Figure 1 Serum neopterin levels in normal controls and patients with chronic hepatitis B and liver cirrhosis.

(0-10), intralobular degeneration and focal necrosis (0-4) and portal inflammation (0-4).

All data was shown in mean \pm SD values. A Chi square test was used to analyze the categorical data, whereas an ANOVA test was used to compare the numerical data of the groups. The homogeneity of the intergroup variance was tested by the Levene method. If the variance was homogenous ($P > 0.05$), then the Tukey test was used, and if not ($P > 0.05$), then the Tamhane test was used to evaluate the significance of the difference between the groups. The correlations between serum neopterin levels and biochemical and histological parameters were determined by the Pearson correlation test. The differences between the groups were taken statistically significant if it is $P < 0.05$.

RESULTS

The characteristics of all the subjects are shown in Table 1. There were no significant differences between the three groups in terms of sex and age. Serum neopterin levels (mean \pm SD) were found to be 14.2 ± 5.6 nmol/L (range 2.7-32) in patients with chronic active hepatitis B, 20.3 ± 7.9 nmol/L (range 12-41) in patients with hepatitis-B-related active liver cirrhosis and 5.2 ± 1.4 nmol/L (range 2.2-6.8) in controls. Serum neopterin levels were significantly elevated in patients with chronic hepatitis B ($P = 0.005$) and liver cirrhosis ($P = 0.008$) than in healthy controls. Patients with liver cirrhosis had higher serum neopterin levels compared to patients with chronic hepatitis B ($P = 0.008$) (Figure 1).

There was a positive significant correlation between serum neopterin levels and ALT levels ($r = 0.41$, $P = 0.004$). No significant correlations were found between serum neopterin levels and AST ($r = 0.18$, $P = 0.22$), GGT ($r = 0.33$, $P = 0.07$), AP ($r = 0.09$, $P = 0.64$), and albumin ($r = -0.34$, $P = 0.06$) levels in patients with chronic hepatitis B. A positive significant correlation was observed between neopterin levels and HAI ($r = 0.52$, $P = 0.001$) (Figure 2A).

According to the Child-Pugh classification, all of the 32 patients with liver cirrhosis were in stage A. In the liver

Table 1 Demographic, biochemical and histological characteristics of all subjects (mean \pm SD)

	Chronic hepatitis B	Liver cirrhosis	Control
<i>n</i>	48	32	40
Sex (M/F)	22/26	15/17	19/21
Age (yr)	8.2 \pm 3.4 (2-17)	7.4 \pm 5.3 (1-16)	7.1 \pm 2.2 (2-16)
ALT (IU/L)	68.8 \pm 52.6 (14-280)	108.9 \pm 76.8 (24-240)	26.1 \pm 7 (14-36)
AST (IU/L)	64.6 \pm 51.4 (21-276)	153.4 \pm 147.6 (39-580)	27.5 \pm 6.7 (18-38)
GGT (IU/L)	23.4 \pm 21.6 (14-162)	115.4 \pm 30.3 (88-439)	36.8 \pm 8.8 (18-48)
AP (IU/L)	528 \pm 193.5 (199-1055)	705.7 \pm 487.4 (228-1851)	167.1 \pm 50.7 (98-240)
Albumin (mg/dL)	3.6 \pm 0.7 (2.5-4.7)	3.2 \pm 1 (2-5.1)	4.4 \pm 0.6 (3.7-5.4)
Neopterin (nmol/L)	14.2 \pm 5.6 (2.7-32)	20.5 \pm 8.6 (13-38)	5.2 \pm 1.4 (2.2-6.8)
HAI	6.2 \pm 2.9 (1-12)	9.1 \pm 2.2 (5-12)	-

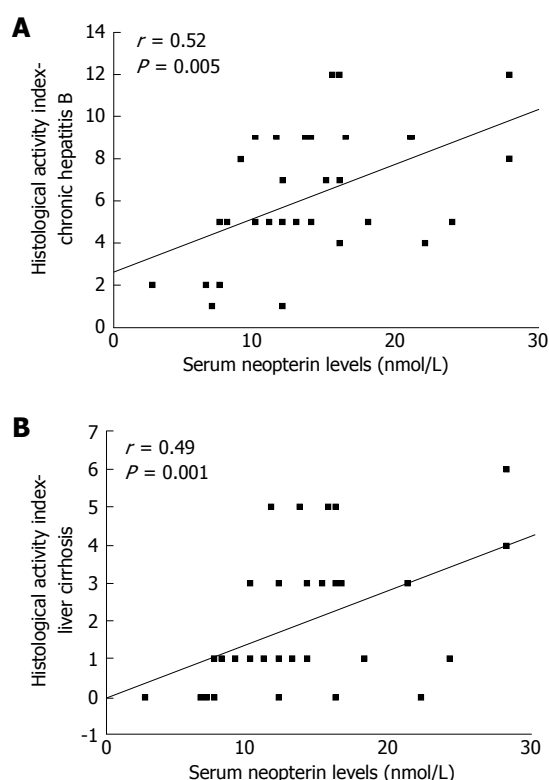


Figure 2 Correlation between serum neopterin levels and histological activity index. A: Chronic hepatitis B; B: Hepatitis-B-related liver cirrhosis.

cirrhosis group, serum neopterin levels were significantly related to ALT values ($r = 0.39$, $P = 0.005$) and HAI ($r = 0.49$, $P = 0.001$) (Figure 2B). There were no correlations between serum neopterin levels and AST ($r = 0.20$, $P = 0.517$), GGT ($r = 0.35$, $P = 0.263$), AP ($r = 0.09$, $P = 0.772$), and albumin ($r = -0.11$, $P = 0.731$) levels.

DISCUSSION

It has been reported that neopterin levels increase in body fluids and change in parallel to the activity of the disease in many infectious diseases and various malign disorders in which activation of the cellular immune system plays an important role in the pathogenesis^[7,16,17]. It has been shown that the activation of cellular immune system stimulates the secretion of γ -interferon from the T lymphocytes, and γ -interferon is an effective inducer of neopterin release by monocytes/macrophages^[10].

Gamma-interferon is produced by the stimulation of T-lymphocytes by several specific antigens, primarily viral antigens, thus it was found that the neopterin levels were especially elevated in viral infections.^[3,17]

Evidence of elevation in neopterin levels in body fluids due to the activation of immune system, which was also supported by several studies involved in diseases leading to activation of the immune system, suggests that elevated neopterin can also be a marker for the follow-up of chronic liver disorders, especially of viral liver disorders^[9]. However, since no such data is available related to children, our results can only be compared to results obtained from the studies carried out with adult patients.

It is suggested that serum neopterin levels can be used as a significant parameter for the differential diagnosis of non-infectious hepatitis and viral hepatitis^[18]. Serum neopterin levels of the patients with acute and chronic hepatitis B were found to be significantly higher than the donors' serum. The relation between neopterin levels and severity of the disease has been proved, and it can be used in combination with clinical data as a prognostic evidence for the progress of the disease^[19]. In asymptomatic HbsAg carriage, acute hepatitis, chronic inactive hepatitis, chronic active hepatitis, liver cirrhosis, hepatocellular carcinoma and alcoholic liver disease, serum and urine neopterin levels were found to be higher than in controls. The most elevated neopterin levels were seen in patients with acute hepatitis^[20].

In adult patients with liver cirrhosis, serum neopterin levels were more elevated than non-cirrhotic patients and control groups^[9,21] whereas out of non-cirrhotic patients, patients with chronic hepatitis B had also elevated neopterin levels^[9].

Serum neopterin levels were elevated in patients with alcoholic cirrhosis^[22]. Neopterin measurement was reported to be beneficial for the differential diagnosis of viral and alcoholic liver diseases, and it has been shown that patients with viral hepatitis had higher neopterin concentrations compared to patients with alcoholic liver diseases^[20].

Serum and urine neopterin levels were elevated from baseline after the initiation of interferon therapy in HbeAg positive patients with chronic hepatitis B, and they remained markedly elevated during the treatment. However, the neopterin levels were restored rapidly to baseline values after the end of the therapy. Therefore

it was suggested that serum and urine neopterin levels could be a good marker of the cellular immunity during interferon treatment in the chronic hepatitis B infection^[23].

In our study, serum neopterin levels was found to be markedly higher in the pediatric patients with chronic hepatitis B and liver cirrhosis than in healthy controls, which is in agreement with the data obtained from the adult patients. It was also higher in patients with cirrhosis when compared with chronic hepatitis B patients. The patients in the cirrhotic stage, independent of their etiology, have elevated concentrations of serum neopterin levels released from the activated macrophages. In those patients, substances that are considered to stimulate the macrophages such as immune complexes or endotoxins, increase in blood due to the lack of peptide clearance by the liver^[24]. These mechanisms explain the highest concentrations of serum neopterin in patients with cirrhosis.

Although no correlation was found between serum neopterin levels and ALT, AST and AP levels in adults with various chronic liver diseases of various etiologies, a negative correlation was found with albumin^[9]. While neopterin levels were found correlated with liver function tests in patients with acute hepatitis, this correlation was not verified in patients with chronic liver diseases^[20]. However, in other studies, a correlation was found between serum neopterin levels and biochemical tests or liver inflammatory grading in patients with chronic hepatitis C and B^[9,25]. We found a significant correlation between serum neopterin levels and ALT or HAI in children with hepatitis-B-related chronic hepatitis B and liver cirrhosis. This data agrees with the data obtained from the adult patients.

In conclusion, these results suggest that measurement of serum neopterin levels can be considered as a marker of inflammatory activity and severity of disease in children with hepatitis-B-related chronic liver disease. However, this needs to be further studied in children.

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RAPID COMMUNICATION

Induction of IgA and sustained deficiency of cell proliferative response in chronic hepatitis C

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against capsid and NS3 revealed a positive recognition mediated by IgG1 in more than 80% of the individuals. On the contrary, less than 30% of the patients showed a positive proliferative response either of CD4+ or CD8+ T cells, being the capsid poorly recognized.

CONCLUSION: These results confirm that while the cellular immune response is narrow and weak, a broad and vigorous humoral response occurs in HCV chronic infection. The observed correlation between IgA and hepatic damage may have diagnostic significance, although it warrants further confirmation.

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Key words: Hepatitis C; Antibody response; Lymphoproliferation; Core; Envelope

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Abstract

AIM: In the present study, antibody and peripheral blood mononuclear cells (PBMC) proliferative responses against hepatitis C virus (HCV) antigens were evaluated in HCV chronically infected patients.

METHODS: Paired serum and PBMC samples were taken six months apart from 34 individuals, either treated or not, and tested by enzyme-linked immunosorbent assay (ELISA) and carboxyfluorescein succinimidyl ester staining.

RESULTS: Over 70% of the patients showed specific IgG and IgM against capsid, E1 and NS3, while HVR-1 was recognized by half of the patients. An increase in the levels of the anti-capsid IgM ($P = 0.027$) and IgG ($P = 0.0006$) was observed in six-month samples, compared to baseline. Similarly, a significantly higher percent of patients had detectable IgA reactivity to capsid ($P = 0.017$) and NS3 ($P = 0.005$) after six months, compared to baseline. Particularly, IgA against structural antigens positively correlated with hepatic damage ($P = 0.036$). IgG subclasses evaluation

INTRODUCTION

Hepatitis C virus (HCV) constitutes a major health problem, since it is infecting an estimated 170 million people worldwide. Probably, the most characteristic feature of this virus is its propensity to cause chronic infection, which is established in the majority of cases and has become a leading indication to liver transplantation in Western countries^[1]. The burden of HCV infection is even more dramatic due to the absence of preventive or therapeutic vaccines. Additionally, the best available treatments, based on pegylated interferon plus ribavirin, are generally effective only in 50% of cases^[2]. Thus, the development of new treatments and prophylactic interventions is currently a priority.

Despite almost two decades of intense research, since its description by Choo and coworkers^[3], correlates of protection have not been entirely established for HCV infection. The specific humoral response to acute infection is considered of relatively low titer and delayed in time from the moment of infection^[4,5]. Antibodies directed to the capsid protein are the first ones to be detected^[4], and have been determined to be principally of IgG and, secondly, of IgM classes in chronic infection^[6]. Particularly, the significance of IgM anti-HCV capsid in chronic infection has been studied^[7,8]. Results indicate that IgM anti-HCV capsid occurrence is directly related to viremia levels^[8] and these antibodies have been found to decrease or disappear in patients in disease remission and increase when the disease reactivates after therapy^[6]. Another feature reported as characteristic of HCV-specific antibody response is its restriction, except for the capsid, to the IgG1 isotype, the rest of the subclasses being poorly prevalent^[4].

Evidence for a significant role of antibody responses in viral clearance seems conflicting, since it has been observed that subjects with antibody deficiencies may spontaneously clear HCV infection^[9]. Several epitopes of the envelope glycoproteins have been identified as targets of neutralizing antibodies^[10]. However, in the majority of the patients, chronic infection is established in spite of their presence^[11], probably due to their absence or low titers in early phases of the infection^[12].

On the other hand, the importance of a sustained, multispecific CD4+, as well as CD8+, T cell responses, targeting numerous epitopes early in infection, has been highlighted^[13-16]. In persistently infected patients, CD4+ and CD8+ T cells are found at low frequencies in peripheral blood, but seem to be compartmentalized in the liver^[17]. It has been demonstrated that, in most subjects, a detectable cell-mediated immune response is generated at the onset of acute infection, but this response progressively disappears in those where HCV infection becomes persistent^[18]. Impaired production of interferon-gamma (IFN- γ) and interleukin- (IL-) 2, as well as incapability to proliferate *in vitro*, have been demonstrated for CD4+ T cells of chronically infected patients^[18-20]. Similarly, CD8+ T cells of persistently infected subjects fail to produce IFN- γ and tumor necrosis factor-alpha (TNF- α) in functional assays^[21,22].

In this work, we aimed at evaluating HCV-specific immune response in chronically infected patients, treated or untreated, using paired blood samples taken 6 months apart. IgG, IgM and IgA levels, as well as IgG1-4 subclasses and peripheral blood mononuclear cells proliferative responses against HCV core, envelope and NS3 antigens were measured. Additionally, we studied the relationship between immune parameters and patients' demographic characteristics.

MATERIALS AND METHODS

Study population

The study cohort included 34 patients with chronic HCV

Table 1 Demographic characteristics of patients with chronic hepatitis C

Characteristic	Value
Age (yr)	
Mean \pm SD	48 \pm 11
Median (Interquartile range)	49 (39-54)
Race (n/%)	
Caucasian	31/91.2
Black	2/5.8
Mixed	1/2.9
Body Mass Index (kg/m ²)	
Mean \pm SD	26.4 \pm 4.5
Median (Interquartile range)	26.4 (23.3-28.9)
Gender (n/%)	
Female	22/64.7
Male	12/35.3
Possible source of infection (n/%)	
Transfusion/surgery	27/79.4
Unknown	7/20.6
Treatment (n/%)	
IFN- α + ribavirin	23/67.6
IFN- α	1/2.9
Untreated	10/29.4
Hepatic damage ¹ (n/%)	
Undetermined	9/26.5
Mild	15/44.1
Moderate	7/20.6
Severe	3/8.8
Alcohol consumption (n/%)	
Yes	4/11.7
No	30/88.2

¹Necro-inflammatory activity.

genotype 1 infection. The enrolment of patients was conducted at the National Institute of Gastroenterology (Havana, Cuba). Written informed consent was obtained from every patient prior to start of the study. All procedures were conducted in accordance with the national ethics guidelines and the Declaration of Helsinki, as revised in 1996. A patient with chronic HCV infection was defined as an individual with detectable HCV RNA and sustained liver injury for more than 6 mo, as monitored by liver function tests [alanine aminotransferase (ALT)/aspartate aminotransferase (AST)] and/or liver biopsy, scored according to the Ishak system. Patients were either treatment naïve or had been treated with the interferon- α (IFN- α) and ribavirin combination during the study period, except one subject who had received IFN- α monotherapy. Blood samples were taken at baseline (T = 0) and 6 mo later (T = 6). Demographic data of patients involved in the study are shown in Table 1.

Antigens

The recombinant proteins Co.120^[23], E1.340^[24] and NS3^[25] are expressed in modified *Escherichia coli* and purified to 90%, except E1.340 which is purified to 85%. E2.680 recombinant protein is expressed in modified *Pichia pastoris* yeast and purified to 85%^[26]. The HVR-1 peptide comprises amino acids 384-414 (TGTYVTGGTAARGVSQFTGLFTSGPSQKIQL) of the E2 protein^[27]. All the recombinant proteins and the

HVR-1 synthetic peptide correspond to a genotype 1b strain. Peptide pools individually comprising the whole sequence of the capsid, E1 and E2 proteins of HCV-1a strain were also used for Peripheral blood mononuclear cells (PBMC) proliferation assays. These peptides were 18 amino acids in length, overlapping adjacent peptides by 10 amino acids. Peptide pools were kindly donated by Dr Naglaa Shoukry (Centre de Recherche du CHUM, Montreal, Canada).

Evaluation of antibody response against HCV antigens

To detect human antibodies to HCV structural antigens, 96-well microtiter plates (Costar, Cambridge, MA, USA) were coated with 100 μ L of Co.120 (10 μ g/mL), E1.340 (10 μ g/mL), HVR-1 synthetic peptide (2 μ g/mL) or NS3 (5 μ g/mL) diluted in coating buffer (50 mmol/L carbonate buffer, pH 9.6) followed by 16-h incubation at 4°C. The wells were washed four times with 0.1% Tween 20 in phosphate buffered saline (0.14 mol/L NaCl, 0.003 mol/L KCl, 0.01 mol/L Na₂HPO₄, 0.001 mol/L KH₂PO₄, pH 7.5) (PBST) and blocked with 200 μ L of PBST containing 2% skim milk (Oxoid Ltd, England) and 5% goat normal serum (blocking solution) for 1 h at 25°C. After four washes with PBST, each well received 100 μ L of a 1:10 dilution of human sera in blocking solution and the plates were incubated at 37°C for 1 h. Sera were diluted 1:80 in blocking solution for the evaluation of the specific response against E1.340. The plates were washed four times with PBST. Then, 100 μ L of horseradish peroxidase-conjugated goat anti-human IgM, IgA or IgG secondary antibodies (Sigma, St Louis, USA), 1:10000, 1:25000 and 1:30000 diluted, respectively, in PBST plus 2% skim milk, were added and the plates were incubated at 37°C for 1 h, followed by four washes with PBST. IgG subclasses were evaluated with the secondary biotinylated antibodies against human IgG1, IgG2, IgG3 and IgG4 (Sigma-Aldrich, St Louis, USA) respectively diluted 1:24000, 1:5000, 1:5000 and 1:1000 in blocking solution. After four washes with PBST, an additional 1 h incubation step at 37°C with extravidin-peroxidase conjugate (Sigma, St Louis, USA), 1:1000 diluted in PBST plus 2% skim milk, was carried out followed by four washes with PBST. In every case, positive reactions were visualized with o-phenylenediamine (Sigma-Aldrich, St Louis, USA) 0.05% in substrate buffer (0.1 mol/L citric acid, 0.2 mol/L NaH₂PO₄, pH 5.0) with 0.015% H₂O₂ (Merck, Germany) as substrate. Reactions were stopped with 50 μ L of 2.5 mol/L H₂SO₄. Measurement of absorbance (*A*) at 492 nm was made in a SensIdent Scan reader plate (Merck, Darmstadt, Germany). At least two human sera, anti-HCV negative by UMELISA (Center for Immunoassay, Cuba), were used as negative controls in each experiment. Anti-HCV positive human sera (as tested by UMELISA, Center for Immunoassay, Cuba), having a known antibody titre of at least 1:150 against the corresponding antigen, served as positive controls. The cut-off value to consider a positive antibody (Ab) response was established as twice the mean *A*_{492nm} of the negative control sera.

PBMC preparation

Blood anticoagulated with acid citrate dextrose (1:9) was processed within 2 h after sample collection. PBMC from HCV patients and a healthy individual were isolated using Ficoll-Paque PLUS density gradients (Amersham, Oslo, Norway), and adjusted to $5-10 \times 10^6$ cells/mL in freezing medium consisting of nine parts of foetal bovine serum (FBS; Hyclone) and one part of DMSO (Sigma, Deisenhofen, Germany). PBMC were stored for 16 h in 1°C freezing containers (Nalgene Nunc International, Rochester, New York, USA) at 80°C and then transferred into liquid nitrogen until use.

Evaluation of CD4+ and CD8+ T cell proliferative response against HCV antigens

T cell proliferation assays were used to analyze HCV specific T cell responses against proteins Co.120, E1.340, E2.680 and NS3 or peptide pools covering core, E1 and E2 HCV proteins, depending on patients' PBMC availability. Cryopreserved PBMC were thawed quickly in a 37°C water bath, and washed twice with R10 medium. After a 16-h resting period at 37°C and 50 mL/L of CO₂, cells were washed twice with PBS, adjusted to 20×10^6 cells/mL and labeled with 4 μ mol/L of carboxyfluorescein succinimidyl ester (CFSE) for 8 min at room temperature in the dark. The reaction was stopped by adding 1 volume of human AB serum (Sigma-Aldrich, St. Louis, USA). Next, PBMC were washed twice with PBS and once with RPMI 1640 medium (Sigma-Aldrich, St. Louis, USA). Cells were finally adjusted to 2×10^6 cells/mL and stimulated or not with the peptide pools (1 μ g/mL) and proteins (2 μ g/mL, except for NS3, of which a concentration of 5 μ g/mL was used) for 6 days at 37°C and 50 mL/L of CO₂. Cells incubated with media alone were considered as negative control. Concanavalin A (ConA, Sigma-Aldrich, St. Louis, USA, 5 μ g/mL) was used as positive control. Cells were harvested, stained with surface antibodies and analyzed by flow cytometry. Anti-CD4 allophycocyanin (APC) (clone # 11 830), anti-CD8 phycoerythrin (PE) (clone # 37 006) and anti-CD8 APC (clone # 37 006) monoclonal antibodies, 4 μ g/mL, 2.5 μ g/mL and 5 μ g/mL respectively, were from R&D Systems (R&D Systems, Minneapolis, USA). The stimulation index (SI) was calculated by dividing the proliferative frequency (%) in the presence of antigen by the proliferative frequency (%) without antigen. The stimulation index was considered positive if ≥ 2.5 after peptide stimulation and ≥ 3 after protein stimulation.

Statistical analysis

GraphPad Prism version 4.00 statistical software (GraphPad Software, San Diego, CA) was generally used to carry out statistical analysis. Unpaired *t* test (for data sets with a Gaussian distribution and equal variances) and Mann Whitney test (for data sets with non-Gaussian distribution or different variances) were used to compare the magnitude of a given response between the two evaluated time points. For comparison of the number of

Table 2 Reactivity of the main antibody classes against core, E1, HVR-1 and NS3 antigens in sera taken at baseline

Isotypes	Percentage of patients with a positive response against the indicated antigen			
	Core	E1	HVR-1	NS3
IgG	91.1	70.8	51.5	88.2
IgM	76.4	78.7	5.8	79.4
IgA	52.9	39.3	12.1	50

Table 3 Reactivity of IgG subclasses against core and NS3 antigens in sera taken at baseline

IgG Subclasses	Percentage of patients with a positive response against the indicated antigen	
	Core	NS3
IgG1	85.2	82.3
IgG2	17.6	39.3
IgG3	11.7	12.1
IgG4	48.4	78.7

positive samples at the two evaluated moments, Fisher's exact test was used. Correlations between variables were analyzed by Spearman's rank correlation coefficient, using SPSS 11.5.1 Software for Windows. Significant differences were considered when $P < 0.05$.

RESULTS

Study subjects

The present study was designed to evaluate the specific immune response against HCV in genotype 1 chronically infected patients, in a 6-mo follow-up period. Of the 34 patients initially enrolled in the study, only 31 could be contacted for a second blood extraction 6 mo later. In this cohort, 73.5% of the patients were over 40 years of age, 67.6% had been treated with the standard combined therapy with IFN- α and ribavirin and only 11.7% of the individuals reported to consume alcohol. Over 73% of the patients had undergone a liver biopsy and of them, 64.7% showed a mild to moderate necro-inflammatory activity, as measured by Ishak scores. Table 1 summarizes the principal demographic characteristics.

Humoral immune response to HCV antigens

The reactivity to HCV antigens was assessed in serum samples from 34 patients chronically infected with HCV. All the patients displayed a positive antibody response against several of the evaluated antigens. At baseline, IgG and IgM reactivities were present in more than 70% of the individuals against Co.120, E1.340 and NS3 proteins (Table 2). Regarding the reactivity towards HVR-1, IgG could only be detected in half of the individuals; still, it was dominant over the rest of the evaluated classes. IgA was generally less frequently detected than IgG and IgM against all antigens.

The assessment of the reactivity of the IgG subclasses, at baseline, against the highly conserved capsid and NS3 antigens, revealed that their recognition

Table 4 Correlation between demographic variables and aspects of humoral response

	Alcohol consumption	Specific treatment	Hepatic damage ¹
IgM ²	$R = -0.53^b$ $P = 0.01$	$R = 0.313$ $P = 0.071$	$R = 0.107$ $P = 0.609$
IgA	$R = -0.402^a$ $P = 0.019$	$R = 0.238$ $P = 0.176$	$R = 0.422^a$ $P = 0.036$
IgG ³	$R = -0.383^a$ $P = 0.028$	$R = 0.257$ $P = 0.149$	$R = 0.100$ $P = 0.635$
IgG4	$R = -0.717^b$ $P = 0.000$	$R = 0.418^a$ $P = 0.014$	$R = 0.401^a$ $P = 0.047$

¹Necro-inflammatory activity; ²IgM positive response to HCV structural antigens; ³IgG positive response to HCV HVR-1 peptide; R = Spearman's correlation coefficient; ^aSignificant correlation at 0.05 level; ^bSignificant correlation at 0.01 level.

was mediated by IgG1 in more than 80% of the patients (Table 3). At the same time, in 78.7% of the individuals a positive IgG4 response could be detected against NS3, while it was detectable only in nearly half of the samples against Co.120. IgG2 and IgG3 were detected in a small percent of the tested samples against both antigens (Table 3).

The reactivity of the main classes IgM, IgA and IgG against Co.120 and NS3 was not only assessed at baseline ($T = 0$), but also 6 months later ($T = 6$) (Figure 1). The comparison of these two time points revealed that there was a significantly higher percentage of individuals with a positive IgA response against both antigens at $T = 6$ (Co.120 52.9% *vs* 88.4%, $P = 0.017$; NS3 50.5% *vs* 88.4%, $P = 0.005$). Interestingly, none of the patients lost the specific IgA reactivity from baseline to the end of the study: instead, the observed increase was totally due to *de novo* responses at $T = 6$. Regarding the magnitude of the response, a statistically significant difference was observed in the IgG and IgM classes against Co.120 (Figure 1A). The mean reactivity was higher at $T = 6$ when compared to baseline (IgM 0.6419 *vs* 0.9099, $P = 0.027$; IgG 0.7802 *vs* 0.9532, $P = 0.0006$).

Correlation analyses between demographic variables and humoral responses revealed that alcohol consumption was negatively correlated with the responses of the main classes IgM ($P = 0.01$), IgA ($P = 0.019$) and IgG ($P = 0.028$), as well as IgG4 ($P = 0.00000182$) (Table 4). On the other hand, positive IgG4 positively correlated with the fact of being treated ($P = 0.014$) and the grade of hepatic damage ($P = 0.047$). Additionally, the hepatic damage, expressed as necro-inflammatory activity, also correlated with IgA ($P = 0.036$), while fibrosis did not ($P = 0.487$).

T cell proliferative response to HCV antigens

PBMC were analyzed in a proliferation assay for their capacity to expand in response to stimulation with HCV antigens. Samples from both $T = 0$ and $T = 6$ were evaluated. Depending on patients PBMC availability, the response to the structural antigens was evaluated by stimulation with the corresponding recombinant protein and a peptide pool. Cells were surface-stained with anti-

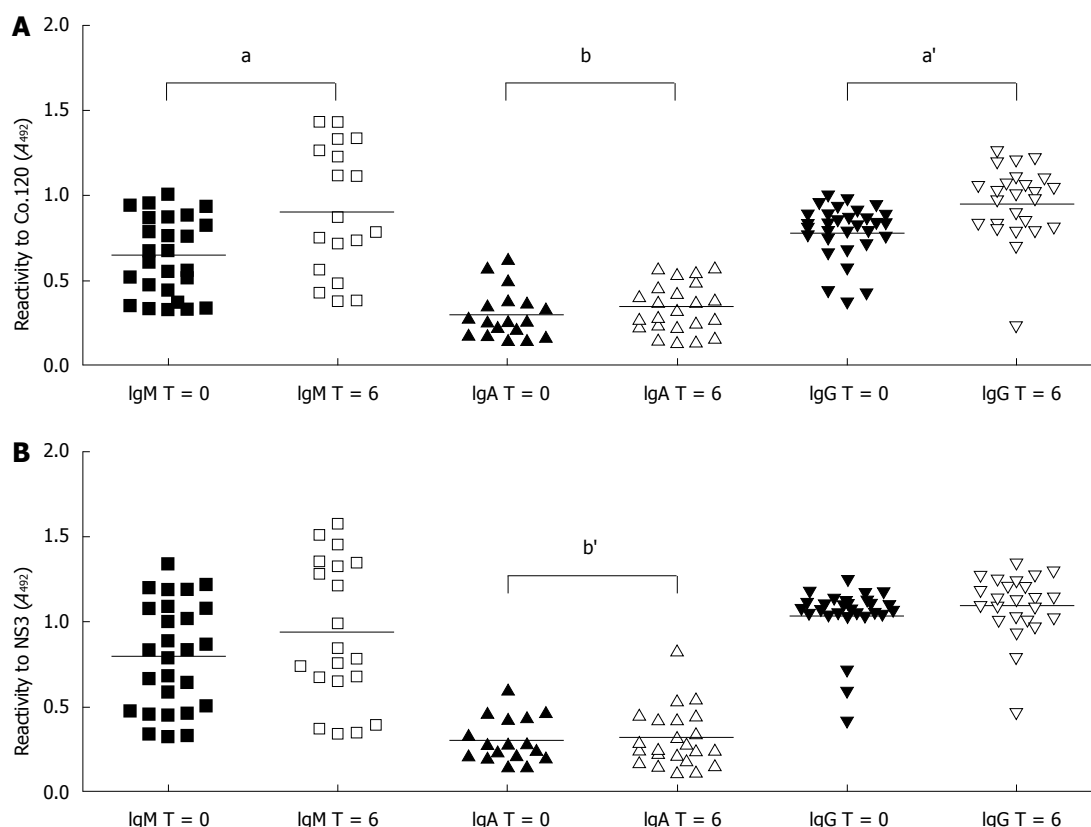


Figure 1 Comparison of antibody reactivity to HCV recombinant Co.120 (A) and NS3 (B) proteins in serum samples from baseline (T = 0) and 6 mo (T = 6) of follow-up. Symbols represent individual values. Only samples showing a positive reactivity are displayed. The horizontal lines represent mean values. Letters over brackets indicate statistical significance (a denotes differences in response magnitude, $^aP = 0.027$, $^{a'}P = 0.0006$; b denotes differences in the number of positive samples, $^bP = 0.017$, $^{b'}P = 0.005$).

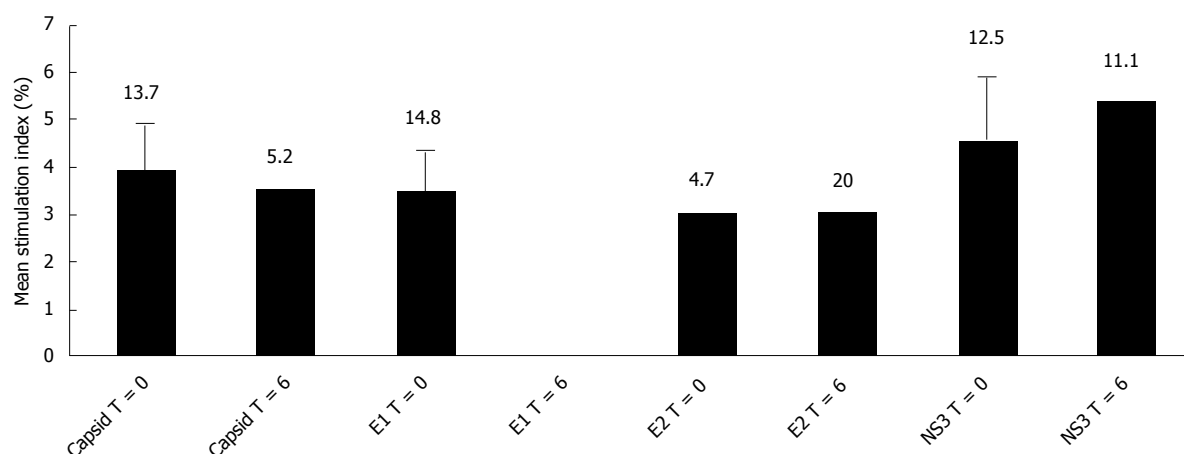


Figure 2 Comparison of CD8+ T cell proliferative response to HCV structural and non-structural antigens in baseline and six months follow up samples. Bars represent mean stimulation index of positive samples only. Error bars represent standard deviation of the mean. Numbers above bars indicate percent of positive samples.

CD4 to evaluate whether the CD4+ cell subpopulation proliferated when stimulated with specific HCV antigens. Particularly, a specific proliferative response was neither detected against the capsid and NS3 proteins at T = 0 nor to E2 at T = 6 (data not shown). Only 10% of the evaluated samples showed a positive response to the capsid at the end of the study, being this antigen the less frequently recognized. The highest percent of positive samples (25%) was detected towards E2 at baseline,

followed by NS3 at T = 6 (20%), while E1 protein was always recognized by 14% of the individuals. None of the patients showed a positive proliferative response against more than one antigen.

On the other hand, the analysis of CD8+ cells allowed the identification of specific responses to all the tested antigens, except to E1 at T = 6 (Figure 2). However, these responses were present in less than 15% of the patients against each antigen. Two patients

showed a positive proliferative response simultaneously against the capsid and E1 proteins at T = 0, while another individual recognized E1 at baseline and also E2, 6 months later. CD8+ cells of only one individual proliferated against all tested antigens (capsid, envelope and NS3 proteins) at T = 0; unfortunately, this patient could not be contacted for a second blood sample, and therefore, the persistence of this peculiar response could not be assessed. Taking into account the results of both CD4+ and CD8+ cell proliferation, we did not detect any patient in which the two time-point samples were consistently positive throughout the study.

DISCUSSION

In this study, we assessed HCV-specific immune responses in a group of HCV chronically infected patients at two different time-points six months apart. The evaluation of the antibody response against viral antigens revealed that all the patients displayed a positive antibody response against several of the evaluated antigens. Specifically, in the majority of patients the reactivity was dominated by IgG and IgM, both in terms of number of positive patients and the magnitude of the response. Particularly, the presence of IgM anti-HCV-capsid antibodies have been regarded as a negative prognostic marker of response to treatment^[28] and a factor associated to recurrence of hepatitis and its severity in HCV-infected liver transplant recipients^[29]. Therefore, their presence in our cohort of chronic patients reinforces the notion of their inefficacy in this phase of the infection. Our results are in agreement with previous works reporting elevated prevalence of both immunoglobulin classes in chronically infected patients^[17,30]. These studies only refer to the specific response to the capsid protein; our results extend this notion to other antigens such as E1 and NS3.

Among the IgG subtypes, IgG1 has been, by far, the most frequently found against all tested viral antigens^[5,30]. Moreover, it has been reported that the antibody response to most HCV antigens is highly restricted to this subclass^[31], the rest of the subclasses being rarely detected. This response restriction to IgG1 has lead studies aiming to find relations of antibody production to long-term outcome after therapy. In fact, it has been observed that IgG1 specific to an N-terminal epitope of the capsid protein decrease in complete responders, while remain unchanged in non-responders. Therefore, these antibodies have been proposed as markers of the efficacy of IFN- α therapy^[30]. Our results showed a high prevalence of IgG1 against the capsid, and also against NS3, HCV's most conserved antigens. Nevertheless, 78.7% of the patients also showed a positive IgG4 response against NS3. It is of general knowledge that in chronic viral infections in humans, viral proteins generally elicit the IgG1 and IgG3 subtypes and to a lesser extent, IgG2 and IgG4. IgG4 has been frequently found dominating in responses to prolonged antigenic stimulation^[32] and has been identified as a major component of circulating immune-complexes in

chronic hepatitis B virus-infected individuals^[33]. Given that antibodies of IgG4 subclass do not activate the complement system through the classic pathway and have a low affinity to Fc- γ receptors, their presence is considered a factor that may contribute to chronicity.

The assessment of correlations between immunological and demographic variables in our study revealed that IgM to structural antigens, IgG to the HVR-1, IgA and IgG4 responses negatively correlated with alcohol consumption, indicating that this habit may dampen the potentiality for generating a diverse immune response. In contrast, the fact of having been treated with the standard therapy positively correlated with the presence of IgG4 responses. Regarding the involvement of IgG4, it has been observed that the nonselective modulatory effect of IFN- α treatment may contribute to widen the diversity of specific IgG subclasses profiles in hepatitis B virus infection, contributing to the high participation of IgG4^[34]. To our knowledge, this effect of antiviral therapy has not been assessed specifically in HCV infection, but seems a plausible hypothesis supporting our findings.

Another positive correlation that could be detected was that between hepatic damage, expressed as necro-inflammatory activity, and IgA response. It has been observed that TGF- β 1, which is produced by hepatic stellate cells and Kupffer's cells, induces the isotype switching to IgA in B lymphocytes proliferating *in vitro*^[35]. This cytokine is a prominent profibrogenic factor during inflammation, tissue regeneration and fibrogenesis^[36] and in line with this, it has been demonstrated that HCV patients have elevated levels of circulating TGF- β 1 *versus* controls^[37]. To our knowledge, whether there is a direct relation between serum IgA and TGF- β 1 circulating levels in HCV chronic patients has not been explored so far. Nevertheless, our results warrant further studies, although they do not point out to a direct correlation with fibrosis, but rather with the necro-inflammatory activity. On the other hand, the liver plays an important role in IgA clearance, and the loss of hepatic function due to chronic inflammation and damage may reduce normal IgA catabolism, and contribute to its accumulation in serum^[38]. Therefore, the observed correlation might be probably indicating that IgA increase is a consequence, rather than a cause, of hepatic damage.

Additionally, IgG4 responses positively correlated with hepatic damage. As previously discussed, IgG4 has not been usually found as a dominant component of HCV specific immune response. Therefore, the implications of the presence of this subclass have not been explored so far in patients chronically infected with HCV. Nevertheless, a series of inflammatory and autoimmune diseases, in which IgG4 have a strong participation, have been described: these are known as IgG4-related sclerosing diseases^[39]. In general, these disorders are characterized by high serum IgG4 levels, which are closely associated with disease activity, in a context of chronic lymphocyte infiltration and fibrosis of the affected organs^[39]. Although the role of

IgG4 in these disorders remains obscure, it has been suggested that the formation and accumulation of immune complexes and the activation of the alternative complement pathway contribute to disease activity^[39]. Further studies with larger cohort of patients are needed to definitively discern the real role of IgG4 in chronic hepatitis C as well as in other chronic inflammatory disorders.

We also evaluated the proliferative response of PBMC of chronic patients against stimulation with HCV antigens, in paired samples taken six months apart. Only a small percent of samples showed a positive response against each of the tested antigens. Moreover, the great majority of the patients displayed a detectable proliferation only to a single antigen and this response was never constant in the two evaluated time points. These results are in agreement with previous works reporting instability, low frequencies and a small number of targeted epitopes by both CD4+ and CD8+ T cells in peripheral blood of chronic patients^[14,16,40]. The characteristic asymptomatic course of this disease hinders the accumulation of immunological data regarding the very early phase of the infection; therefore, it has been difficult, so far, to discriminate between primary T cell failure and early T cell exhaustion or deletion, once chronic infection has already been established. In fact, both phenomena seem to operate in different patients and equally lead to persistence^[41]. Many mechanisms are postulated to be involved in T cell failure, namely impaired antigen presentation^[42,43], reduced cytokine secretion by antigen presenting cells^[44], immunomodulatory effects exerted by different viral factors^[45,46] and increased levels of CD4 + CD25^{high} Treg cells^[47].

In summary these results confirm that in patients chronically infected with HCV, either naïve or non-responders to the standard therapy with IFN- α plus ribavirin, cellular proliferative responses are rarely detected, usually weak, not sustained and narrowly directed. On the other hand, the humoral response is characterized by a broad representation of antibody classes and subclasses, some of which have not been demonstrated to contribute to viral clearance, but rather to persistence. Particularly, the association of specific IgA response to necro-inflammatory activity paves the way to further studies to confirm its utility as an easy-to-measure marker of increased histological activity.

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COMMENTS

Background

Correlates of protection against hepatitis C virus (HCV) are extensively pursued in nowadays research. Early, vigorous and sustained peripheral blood mononuclear cells (PBMC) proliferative responses specific to HCV have been regarded as pivotal for viral clearance. On the other hand, antibody responses' contribution is still controversial and the significance of specific antibody classes during chronic infection has been investigated.

Research frontiers

So far, in HCV infection, the most extensively studied antibodies are those directed to the capsid protein. Several data indicate that IgM anti-HCV capsid occurrence is directly related to viremia levels. Additionally, HCV-specific antibody response is regarded as restricted to the IgG1 isotype, except for the capsid. The rest of the classes and IgG subclasses have been found very rarely represented, and therefore their significance in acute and chronic HCV is unclear.

Innovations and breakthroughs

Correlation analysis between demographic variables and humoral response confirmed the negative influence of alcohol consumption on the immune response, particularly on responses of the main immunoglobulin classes. On the other hand, IgG4, an IgG subclass characteristic of chronic antigenic stimulation, positively correlated with the grade of necro-inflammatory activity and the fact of being treated with the standard therapy; the latter already demonstrated for hepatitis B virus (HBV), but not for HCV. Additionally, for the first time a positive correlation between necro-inflammatory activity with HCV-specific IgA was found.

Applications

Particularly, the association of specific IgA response to necro-inflammatory activity paves the way to further studies to confirm its utility as a non invasive, easy-to-measure marker of increased histological activity in chronic HCV infection.

Peer review

An interesting study assessing the humoral response in chronic HCV patients. The association of elevated IgG and globulins with fibrosis have been described as referenced. It is an interesting observation to find IgA specific to HCV antigens.

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Hepatoma cells up-regulate expression of programmed cell death-1 on T cells

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Abstract

AIM: To investigate the effect of hepatoma cells on up-regulation of programmed cell death-1 (PD-1), and the function of PD-1 on T cells.

METHODS: HepG2 or HepG2.2.1.5 cells were co-cultured with a lymphoma cell line-Jurkat cells. PD-1 expression was detected by flow cytometry. IL-2, INF- γ and IL-10 in culture supernatant were detected by enzyme-linked immunosorbent assay (ELISA). Cytotoxic action of T cells was determined by MTT reduction assay-direct mononuclear cell cytotoxicity assay.

RESULTS: The PD-1 expression on Jurkat cells increased by $16.17\% \pm 2.5\%$ and $17.43\% \pm 2.2\%$ after HepG2 or HepG2.2.1.5 cells were co-cultured for 48 h. The levels of IL-2, INF- γ and IL-10 in the culture supernatant were 202.9 ± 53.0 pg/mL, 88.6 ± 4.6 pg/mL and 63.7 ± 13.4 pg/mL respectively, which were significantly higher than those (102.9 ± 53 pg/mL, 39.3 ± 4.2 pg/mL, and 34.6 ± 13.7 pg/mL) in the control group ($P < 0.05$). The OD value for MTT assay in the blocking group (0.29 ± 0.06) was significantly higher than that (0.19 ± 0.09) in the control group ($P < 0.05$).

CONCLUSION: PD-1 expression on Jurkat cells is up-regulated by hepatoma cells, cytokines and cytotoxic action are elevated after PD-1/PD-L1 is blocked.

INTRODUCTION

Programmed cell death-1 (PD-1), a member of the CD28 family, was first isolated from T cell hybridoma by subtractive hybridization in 1992^[1], and is expressed on activated T and B cells^[2]. PD-1 has been recently found to play a role in immunity regulation as an inhibitory co-signaling molecule^[3]. Upon its ligands (PD-L1 and PD-L2) are ligated, PD-1 decreases T cell receptor (TCR)-mediated proliferation, cytokine production and cytolytic activity^[4-7]. Barber *et al*^[8] showed that up-regulation of PD-1 expression on T cells is associated with the exhaustion of T cells in lymphocytic choriomeningitis virus (LCMV) infection. The number of effective T cells increases and their function markedly improves when the interaction of PD-1 and PD-L is blocked. It was reported that PD-1 also plays a significant role in some autoimmune diseases^[9-11] and viral infectious diseases, especially in chronic infectious diseases caused by human immunodeficiency virus (HIV)^[12-14], hepatitis C virus (HCV)^[15,16] and hepatitis B virus (HBV)^[17-19]. There is evidence that PD-1 suppresses immune activation in PD-1-deficient mice with autoimmune disease^[20]. Single-nucleotide polymorphisms at the PD-1 locus have also been identified in patients with autoimmune disorders such as systemic lupus erythematosus, rheumatoid arthritis, and type 1 diabetes^[21].

Recent evidence from studies on tumors shows that the PD-1/PD-L pathway might play a critical role in tumor immunity evasion^[22-24]. PD-1 is up-regulated on tumor specific T cells and PD-L is up-regulated in tumor tissue^[25-27]. As a result, the function of tumor specific T cells is suppressed, leading to the immune escaping of tumor cells, and the level of mRNA and PD-L1

protein can be detected in tumor tissues^[25-27]. In addition, PD-1/PD-L interaction promotes apoptosis of T cells, induces clearance of specific T cells, and ultimately inhibits the anti-tumor immunity response^[27]. Blocking the interaction of PD-1/PD-L with anti-PD-1 antibody partially recovers the function of tumor specific T cells.

It was reported that the expression of PD-L can be induced by virus and cytokines such as IFN- α and INF- γ ^[28]. However, no evidence for the induced expression of PD-1 is available. Liver, an important immune organ, plays a critical role in immune regulation although it is prone to induce immune tolerance in many cases. The present study was designed to investigate whether hepatoma cells (HepG2 and HepG2.2.1.5) induce expression of PD-1 in T cells. The functional role of PD-1/PD-L interaction was also studied.

MATERIALS AND METHODS

Cell culture

Jurkat cells were cultured in RPMI 1640 medium (Gibco, USA), supplemented with 10% fetal calf serum, 300 μ g/mL glutamine, 100 U/mL penicillin and 100 μ g/mL streptomycin. HepG2.2.1.5 cells or HepG2 cells provide by Mountsinai Medical Center were cultured in complete DMEM (Gibco, USA) containing 380 μ g/L G418.

Co-culture system

HepG2 and HepG2.2.1.5 cells were cultured in 6-well plates (5×10^5 cells/well) for 24 h and Jurkat cells (5×10^5 cells/well) were added and co-cultured for 48 h. The suspended cells were collected for analysis of PD-1. Jurkat cells were cultured solitarily for 48 h as controls.

Analysis of PD-1 expression

Cell surface expression of PD-1 was detected by flow cytometry after incubated with allophycocyanin (APC)-conjugated anti-PD-1 antibodies (eBioscience). Cells were collected and suspended in PBS containing 1% fetal calf serum (FCS). The cell density was adjusted to 1×10^6 cells/vial. After incubated with anti-PD-1 antibodies or matching isotype controls at 37°C for 30 min, cells were washed with PBS and fixed in 2% paraformaldehyde for analysis. A total of 20000 gated cells were analyzed on Becton Dickinson FACS (Becton Dickinson, USA) using the CELLQuest™ software.

Blockade of PD-1/PD-L1 interaction

Antibody against human PD-L1 (eBioscience) was used to block PD-L1. Jurkat cells were activated with phytohemagglutinin (PHA) (2 μ g/mL, Sigma, USA) and co-cultured with HepG2 or HepG2.2.1.5 cells. Anti-PD-L1 antibodies (25 μ g/mL) were added into the culture to block the interaction of PD-1 and PD-L1 for 48 h. Mouse IgG, as a control antibody, was used in the control group. After cultured for 48 h, the supernatants of co-cultures were collected and stored at -80°C.

Analysis of cytokine secretion

Table 1 Up-regulated expression of PD-1 on T cells ($n = 24$, mean \pm SD)

Groups		PD-1(%)
Controls		0.70 \pm 0.03
	HepG2	16.17 \pm 2.5 ^a
Co-cultured	HepG2.2.1.5	17.43 \pm 2.2 ^b

^a $P = 0.000$, ^b $P = 0.000$ vs control group.

Levels of IL-2, IL-10 and INF- γ in the supernatants were measured by ELISA (Ucytech, Netherlands) following its manufacturer's instructions.

Analysis of cytolytic activity

Cytolytic activity of Jurkat cells was detected by MTT assay. After co-cultured for 48 h, Jurkat cells were isolated and MTT was added to incubate HepG2.2.1.5 cells for 4 h, and bleached with DMSO. Finally, the cytolytic effect of Jurkat cells on HepG2.2.1.5 cells was analyzed on Bio-Rad 450 (USA).

Statistical analysis

Results were expressed as mean \pm SD or percentage. Comparison between groups was made using Student's unpaired *t*-test. $P < 0.05$ was considered statistically significant. All analyses were performed using SPSS 13.0 for Windows.

RESULTS

Enhancement of PD-1 expression on Jurkat cells

PD-1 expression on Jurkat cells was determined by FACS analysis at 48 h after co-cultured with HepG2 or HepG2.2.1.5 cells. Jurkat cells were also cultured solitarily as controls. The expression of PD-1 was induced on Jurkat cells after co-culture with HepG2 or HepG2.2.1.5 cells for 48 h, which was significantly higher on Jurkat cells co-cultured with hepatoma cells than on controls ($P = 0.000$, Table 1).

Function restoration of T cells

Supernatants were collected from the blocking and control groups. To investigate the influence of cytokine production after PD-1/PD-L1 was blocked, the levels of IL-2, IL-10 and INF- γ were measured. After PD-L1 was blocked with specific antibodies, the levels of IL-2, IL-10 and INF- γ were much higher in the blocking group than in the control group ($P = 0.000$, Table 2).

Furthermore, the effect of the PD-1/PD-L1 pathway on cytolytic activity of T cells was also investigated by MTT assay. The *A* value (0.29 ± 0.06) in the blocking group was much higher than that (0.19 ± 0.09) in the control group ($P = 0.000$).

DISCUSSION

Activation of resting lymphocytes triggers expression of several products of the immunoglobulin superfamily of genes. These activation-induced antigens are involved in

Table 2 Secretion of cytokines by T cells (pg/mL, *n* = 24, mean \pm SD)

Groups	IL-2	IFN- γ	IL-10
Control group	102.9 \pm 53.0	39.3 \pm 4.2	34.6 \pm 13.7
Blocking group	202.9 \pm 53.0 ^c	88.6 \pm 4.6 ^d	63.7 \pm 13.4 ^e

^c*P* = 0.000, ^d*P* = 0.000, ^e*P* = 0.000 *vs* control group.

many physiological and pathological processes including cell proliferation (IL-2R), functional differentiation (CTLA-4), and apoptosis (Fas)^[29,30]. The expression patterns of these antigens are cell-specific, and have different regulation functions in different cells. PD-1, a member of the CD28 family, which was isolated from apoptosis-induced T cell hybridoma in 1992^[1], is expressed on activated T and B cells^[2].

Agata *et al*^[2] showed that PD-1 expresses on activated T and B cells. Anti-CD3 and concanavalin A (ConA) can stimulate its expression on thymocytes and T cells in spleen, and anti-IgM antibody can stimulate its expression on B cells in spleen. Vibhakar *et al*^[31] also demonstrated that PD-1 mRNA and protein levels in Jurkat cells are up-regulated in a time-dependent manner during phorbol ester (TPA)-induced differentiation, indicating that lymphocyte activators can up-regulate PD-1 expression on lymphocytes. Since PHA is another T cell activating agent, the expression of PD-1 in T cells can be detected after stimulation of PHA. A time-dependent up-regulation of hPD-1 was also observed during PHA induction (data not shown), and was used as a stimulus of Jurkat cells in our blocking experiment.

It was reported that, as an inhibitory co-stimulating molecular, PD-1 plays a role in immune regulation and is associated with the exhaustion of effective T cells^[32-34]. Barber *et al*^[8] showed that, in chronic viral infection diseases, PD-1 is highly expressed on the exhausted LCMV-specific CD8 T cells and blocking the PD-1/PD-L1 interaction during the chronic phase of infection can efficiently reanimate the exhausted CD8 T cells and promote clearance of the persisting virus. In contrast, PD-1 expression is transiently induced and declines quickly to its basal level in acute LCMV-Armstrong infection, thus promoting studies on other diseases associated with immune. Up-regulation of PD-1 expression on effective T cells leads to suppression of immune, which might be the underlying mechanism of immune evasion. PD-L1/PD-L2 expression in a variety of tumor cells has been detected in human tumors^[35,25-27], while PD-1 over-expression on tumor specific T cells has also been observed^[23]. Interaction of PD-1/PD-L1 promotes apoptosis of T cells, inhibits anti-tumor immune response of T cells, and stimulates growth of tumors^[27]. Obstructing the interaction of PD-1/PD-L1 enhances the function of T cells, hampers development of tumors^[22,23,26]. In the present study, tumor cells induced expression of PD-1 on T cells. After co-cultured with hepatoma cells, PD-1 was expressed on T cells, but not expressed on Jurkat cells after cultured for 48 h solitarily. PD-1 was expressed on T cells after Jurkat

cells were co-cultured with the supernatant of hepatoma cells (data not shown), suggesting that hepatoma cells can up-regulate the expression of PD-1.

These findings lead to the clinical use of PD-1 blockers in the treatment of tumors. In chronic infectious diseases, virus can induce the expression of PD-1 on T cell. However, the precise mechanism PD-1 blockers still remains unclear. We observed the effect of tumor cells on PD-1 expression in T cells. Jurkat cells, a kind of CD4⁺ T cells, can be used as target cells co-cultured with hepatoma cells. FACS analysis showed that tumor cells could induce PD-1 expression on T cells. In this study, HepG2.2.1.5 cells transferring HBV genome and HepG2 cells not transferring HBV genome could induce PD-1 expression, suggesting that HBV has no effect on PD-1 expression on T cells.

In addition, the function of PD-1 on T cells was also observed. Anti-PD-L1 antibody was used to block the interaction of PD-1 and PD-L1. PD-1 was induced by PHA, and PD-L1 was expressed in HepG2.2.1.5 cells identified by FACS (data not shown). Jurkat cells after activated by PHA were co-cultured with HepG2.2.1.5 cells. Antibodies against human PD-L1 were added into the co-culture system as a blocking group, while mouse IgG was added as a control group. The levels of cytokines including IL-2, INF- γ and IL-10 in culture supernatant and the cytolytic activity of T cells were detected, which were significantly elevated in blocking group compared to the control group, suggesting that both Th1 and Th2 have immune responses can be restored and PD-1/PD-L that negatively regulates the immune reaction by blocking the PD-1/PD-L pathway can recover the function of T cells, which introduces a new theory of tumor immune evasion. This new mechanism of tumor immunology might provide a novel target for therapy.

COMMENTS

Background

Programmed cell death-1 (PD-1), originally isolated from apoptotic T cells, is a 55-kDa transmembrane protein with an extracellular IgV-like domain and a 97-amino acid cytoplasmic tail containing an immunotyrosine inhibitory motif (ITIM) and an immunotyrosine switch motif (ITSM). PD-1 has two ligands: PD-L1 and PD-L2, which are members of the CD28/B7 superfamily. The expressions of CD28, CTLA-4, and ICOS are limited in T cells. PD-1 can be expressed on activated T, B and myeloid cells. The expression of PD-L1 has been detected in many organ tissues, such as heart, lung, pancreas, muscle, and placenta, including lymphocytes and non-lymphocytes. The expression of PD-L2 is restricted in DC and macrophages.

Research frontiers

Recent findings suggest that PD-1/PD-L pathway plays a role in regulating tolerance and autoimmunity. The role of PD-1 and its ligands in regulating human autoimmune disease, infectious diseases and tumors has been investigated. Interaction of PD-1 and PD-L has been found to be important for controlling effective T cells. Significantly increased expression of PD-1 and PD-L1 in T and B macrophages/dendritic cells and tumor cells, associated with T-cell exhaustion and disease progression, immobilized auto-antibodies to PD-L, can stimulate T cell proliferation, cytokine production, and programmed cell death. The up-regulation of PD-L1 in tumors and PD-1 in T cells can lead to immune tolerance.

Innovations and breakthroughs

The present study demonstrated the effect of hepatoma cells on up-regulating

the expression of PD-1 in T cells. In addition, the function of PD-1 in T cells was assessed, showing that the cytokine production and cytotoxicity of T cells can be elevated by blocking the interaction of PD-1 and PD-L1.

Applications

The up-regulation of PD-1 in T cells by hepatoma cells has led to a new hypothesis that the PD-1 and PD-L pathway may be a means by which tumors evade T cell recognition. Manipulation of the PD-1 pathway can enhance immune response, and may become a novel strategy for the treatment of tumors.

Terminology

MTT is a direct mono-nuclear cell direct cytotoxicity assay used to detect the cytotoxicity of T cells. PHA is a T cell stimulus that can activate T cells. G418, a kind of antibiotics, is used in selective cell culture medium. G418 must be added into culture medium of HepG2.2.1.5 cells to support the selective survival of HepG2.2.1.5 cells.

Peer review

This is a very interesting study, showing that HCC-induced modulation of PD-1 expression in T-cells might contribute to immune evasion. This concept might hold its promise for new therapeutic interventions. The experiments support the authors' claim.

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RAPID COMMUNICATION

Effects of recombinant human growth hormone on enterocutaneous fistula patients

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Abstract

AIM: To explore the effects of recombinant human growth hormone (rhGH) on intestinal mucosal epithelial cell proliferation and nutritional status in patients with enterocutaneous fistula.

METHODS: Eight patients with enterocutaneous fistulas received recombinant human growth hormone (10 µg/d) for 7 d. Image analysis and immunohistochemical techniques were used to analyse the expression of proliferating cell nuclear antigen (PCNA) in intestinal mucosal epithelial cells in biopsy samples from the patients who had undergone an endoscopic biopsy through the fistula at day 0, 4 and 7. Body weights, nitrogen excretion, serum levels of total proteins, albumin, prealbumin, transferrin and fibronectin were measured at day 0, 4 and 7.

RESULTS: Significant improvements occurred in the expression of PCNA in the intestinal mucosal epithelial cells at day 4 and 7 compared to day 0 ($24.93 \pm 3.41\%$, $30.46 \pm 5.24\%$ vs $12.92 \pm 4.20\%$, $P < 0.01$). These changes were accompanied by the significant improvement of villus height (500.54 ± 53.79 µm, 459.03 ± 88.98 µm vs 210.94 ± 49.16 µm, $P < 0.01$), serum levels of total proteins (70.52 ± 5.13 g/L, 74.89 ± 5.16 g/L vs 63.51 ± 2.47 g/L, $P < 0.01$), albumin (39.44 ± 1.18 g/L, 42.39 ± 1.68 g/L vs 35.74 ± 1.75 g/L, $P < 0.01$) and fibronectin (236.3 ± 16.5 mg/L, 275.8 ± 16.9 mg/L vs 172.5 ± 21.4 mg/L, $P < 0.01$) at day 4 and 7, and prealbumin (286.38 ± 65.61 mg/L vs

180.88 ± 48.28 mg/L, $P < 0.05$), transferrin (2.61 ± 0.12 g/L vs 2.41 ± 0.14 g/L, $P < 0.05$) at day 7. Nitrogen excretion was significantly decreased at day 7 (3.40 ± 1.65 g/d vs 7.25 ± 3.92 g/d, $P < 0.05$). No change was observed in the body weight.

CONCLUSION: Recombinant human growth hormone could promote intestinal mucosal epithelial cell proliferation and protein synthesis in patients with enterocutaneous fistula.

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Key words: Recombinant human growth hormone; Enterocutaneous fistula; Intestinal; Epithelial cell; Proliferating cell nuclear antigen

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Gu GS, Ren JA, Li N, Li JS. Effects of recombinant human growth hormone on enterocutaneous fistula patients. *World J Gastroenterol* 2008; 14(44): 6858-6862 Available from: URL: <http://www.wjgnet.com/1007-9327/14/6858.asp> DOI: <http://dx.doi.org/10.3748/wjg.14.6858>

INTRODUCTION

From the mid 1980s, recombinant human growth hormone (rhGH) has been applied clinically. Growth hormone is a peptide hormone which stimulates proliferation and differentiation of many kinds of cells. It also has anabolic effects on the modulation of energy and substance metabolism. Previous animal experiments^[1-3] have demonstrated it could promote the structural repair of the intestinal mucosa in short bowel rats, but few studies have made direct observations of the effects of rhGH on intestinal mucosa in human. The objective of this study was to explore the effects of rhGH on intestinal mucosal proliferation and nutritional status in patients with enterocutaneous fistula.

Table 1 General state of the patients

Age (Yr)	Sex	Weight (kg)	Distance from ligament of tretiz (cm)	Rest energy expenditure (KJ)	Enteral nutrition energy (KJ)
65	F	52	40	4276	6276
42	M	68	80	5648	8368
30	M	80	100	6192	9205
32	M	61	120	4384	6276
18	M	48	60	4322	6276
72	F	56	90	5773	8368
38	M	77	135	6158	9205
27	F	49	85	4359	6276

MATERIALS AND METHODS

Study protocol

Eight patients (Table 1) with enterocutaneous fistula were injected with rhGH (10 U/d) for 7 d. Intestinal mucosa biopsies were performed by endoscopy through the fistula at 20 cm proximal to the fistula at day 0, 4 and 7. All the patients gave informed consent to participate in the study. This study was approved by the Ethical Committee of Jinling Hospital, Nanjing University. Biological tests revealed no signs of inflammation, metabolic disturbances or hepatic, renal and cardiac dysfunction before the patients were enrolled into the study. The subjects had a mean body mass index of 14.37 kg/m² (range 11.09-17.65 kg/m²). For all the subjects, enteral nutrition (Peptisorb, Nutricia, Holland) was prescribed and taken by nasogastric or nasointestinal tube to maintain the metabolic balance. The formula contained 1 kcal/mL, and the total calories given according to the energy expenditure was determined by indirect calorimetry (MedGraphics, USA). Endoscopic biopsies were fixed in formalin for histological assessment.

Recombinant human growth hormone (rhGH)

rhGH (Saizen) was provided by Serono China Pte. Ltd, China. The dose of rhGH was 10 U/d administered once a day (8:00 pm) as a subcutaneous injection to an upper limb, beginning on day 1 and continued for 7 d.

Immunohistochemical staining

To assess the degree of cell proliferation, an immunohistochemical technique based on the proliferating cell nuclear antigen (PCNA) was used. Sections from tissue samples were dewaxed, taken through alcohol and then immersed for 10 min in 25% phosphate-buffered saline in methanol with 0.3% hydrogen peroxide to block endogenous peroxidase activity. Sections were subsequently taken to water and immunostained using the Vectastain ABC peroxidase kit (Vecta Laboratories, Burlingame, CA). 0.4% diaminobenzidine (DAB, Aldrich Co.) was employed as a chromogen and a light haematoxylin counterstain was used. Counts were carried out in 30 crypts per preparation under microscope (40 ×), using an automatic image analysis system (HPLAS-1000, Tongji qianping Ltd). A proliferation index was determined based on the

Table 2 Changes of intestinal mucosal villus height, PCNA and nitrogen excretion before and after treatment with rhGH

	The days when treated with rhGH		
	0	4	7
Villus height (μm)	210.94 ± 49.16	500.54 ± 53.79 ^b	459.03 ± 88.98 ^b
PCNA labelling index (%)	12.92 ± 4.20	24.93 ± 3.41 ^b	30.46 ± 5.24 ^b
Nitrogen excretion (g/d)	7.25 ± 3.92	4.64 ± 1.95	3.40 ± 1.65 ^a

All values are expressed as mean ± SD. ^a*P* < 0.05, ^b*P* < 0.01 vs day 0.

ratio between PCNA-positive cells and the total number of cells per longitudinal crypt section at the base of the crypt. This index is equal to the quotient of the number of proliferating cells and the total number of cells multiplied by 100.

Mucosal height

Sections from tissue samples were fixed in 4% paraformaldehyde, dehydrated with alcohol and then paraffin-embedded. The formatted specimens were cut by sliding microtome and stained with hematoxylin and eosin. Samples were analyzed with the automatic image analysis device (HPLAS-1000, Tongji qianping Ltd), using a microscope at 10 ×. The total mucosal height from the base of the crypt to the villous tip was measured (10 measures per preparation, in the 10 highest villi of each sample, and the base of the crypts measurement reached the muscularis mucosae).

Biochemical assays and nitrogen excretion

Serum albumin, prealbumin, transferrin and fibronectin concentrations were determined by automatic biochemical analysis device (Beckman Coulter, USA).

Daily urinary and fecal nitrogen excretion was determined by the Kjeldahl method at day 0, 4 and 7.

Statistical analysis

Data were analyzed using a statistical software package for Windows (SPSS version 10.0, SPSS Inc, Chicago, IL, USA). All variables of each group were described by common statistical methods. Results are presented as mean ± SD. One-way ANOVA for repeated measures was performed in order to evaluate the differences among the three states of the study. The level of significance was set at *P* value of 0.05 or less.

RESULTS

Villus height and proliferative activity

Compared with the baseline, significant improvement occurred in the intestinal mucosal villus height at day 4 and 7 (both *P* < 0.01), which was accompanied by the increase of proliferative activity of epithelial cells assessed by the PCNA labelling index (both *P* < 0.01) (Table 2, Figure 1).

Nitrogen excretion, body weight and serum levels of protein

Nitrogen excretion was significantly decreased at day 7 (*P*

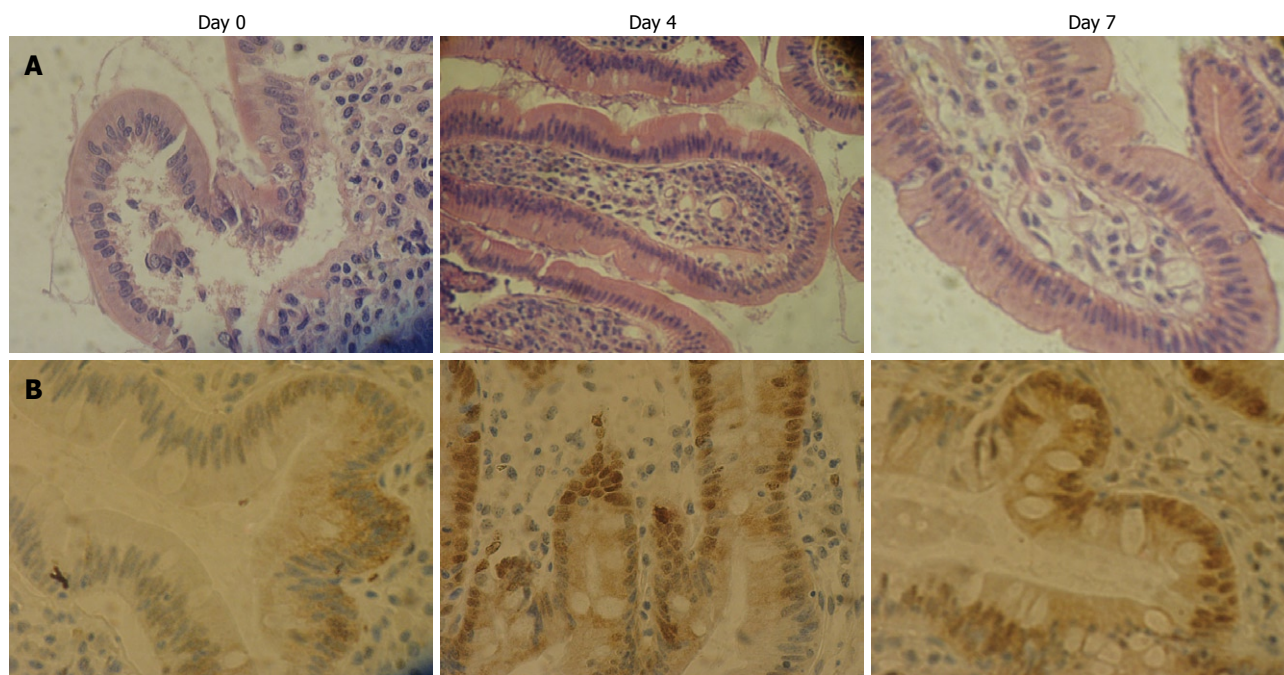


Figure 1 Villus height and proliferative activity. Significant improvements occurred in villus heights and in the expression of PCNA on the intestinal mucosal epithelial cells at day 4 and 7 ($P < 0.01$). A: Villus heights; B: PCNA labelling index.

Table 3 Changes in body weight and serum proteins before and after treatment with rhGH

	The days when treated with rhGH		
	0	4	7
Body weight (kg)	37.39 ± 12.48	38.64 ± 12.84	39.13 ± 12.19
Body mass index (kg/m ²)	14.37 ± 3.28	15.21 ± 3.41	15.40 ± 3.24
Total proteins (g/L)	63.51 ± 2.47	70.52 ± 5.13 ^b	74.89 ± 5.16 ^b
Albumin (g/L)	35.74 ± 1.75	39.44 ± 1.18 ^b	42.39 ± 1.68 ^b
Prealbumin (mg/L)	180.88 ± 48.28	231.38 ± 52.31	286.38 ± 65.61 ^a
Transferrin (g/L)	2.41 ± 0.14	2.49 ± 0.12	2.61 ± 0.12 ^a
Fibronectin (mg/L)	172.5 ± 21.4	236.3 ± 16.5 ^b	275.8 ± 16.9 ^b

All values are expressed as mean ± SD. ^a $P < 0.05$, ^b $P < 0.01$ vs day 0.

< 0.05) (Table 2). Serum levels of total proteins, albumin and fibronectin were significantly increased at day 4 and 7 (both $P < 0.01$). The levels of prealbumin and transferrin were increased at day 7 ($P < 0.05$) (Table 3). No change was observed in the body weight.

DISCUSSION

Previous studies have shown that GH stimulates bowel growth^[1-4]. Administration of GH improves gut mucosal structure in animals with short bowel syndrome^[5-7] and may promote the structural repair of the graft^[8,9]. Experiments *in vitro* have also demonstrated that GH is involved in the regulation of crypt cell proliferation in the human small intestine^[10-13].

The therapeutic efficacy of rhGH has been suggested by results of animal studies. In the present study the effects of rhGH *in situ* on the intestine of patients with enterocutaneous fistula were observed. Our results revealed that significant improvement occurred in the intestinal mucosal villus height at day 4 and 7, which was

accompanied by the increase of proliferative activity of epithelial cells assessed by the PCNA labelling index.

Nutrient malabsorption often occurs in patients with gastrointestinal fistula^[14-17], and it causes body weight loss, barrier damage, followed by bacterial translocation from the gastrointestinal tract to the mesenteric lymph nodes, and even blood. Administration of glutamine and growth hormone synergistically reduces bacterial translocation in sepsis^[18,19]. Hormonal therapy with GH can improve weight gain in a rat model of severe short bowel syndrome. This improvement in weight gain was associated with an increase in nutrient transport at the cellular level and variable increases in villus size^[20,21]. GH treatment increased [¹⁴C] glucose and [³H] palmitic acid plasma concentration after oral nutrient tolerance tests^[22]. Clinical trials also showed that GH could promote positive nitrogen balance and protein synthesis^[23-25]. However, there are some conflicting results: no improvement was observed in the absorption of total energy, carbohydrate, fat, nitrogen, or wet weight of stool or stool electrolytes compared with baseline and placebo measurements^[26-28]. In the present study the body weights of the eight patients were maintained at normal level. All the patients showed positive nitrogen balance and the nitrogen excretion was significantly decreased at day 7. Serum levels of total proteins, albumin and fibronectin were significantly increased at day 4 and 7. And the levels of prealbumin and transferrin were increased at day 7.

GH stimulated the formation and deposition of collagen in both skin incisional wounds and in colonic anastomoses in rats^[29,30].

After the trial all the eight patients underwent surgery to close the fistula and they recovered very well, and no fistula recurred.

In summary, our study shows that rhGH can promote intestinal mucosal epithelial cell proliferation and protein synthesis in patients with enterocutaneous fistula.

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COMMENTS

Background

In some medical literature, hormonal therapy with GH has been shown to improve weight gain in a model of severe short bowel syndrome. This improvement in weight gain was associated with an increase in nutrient transport at the cellular level and variable increases in villus size. But there are some conflicting results: no improvement was observed in the absorption of total energy, carbohydrate, fat, nitrogen, or wet weight of stool or stool electrolytes compared with baseline and placebo measurements.

Research frontiers

This study has been carefully designed to investigate whether recombinant human growth hormone (rhGH) could increase the proliferative activity of epithelial cells and nutrient absorption in human. The results showed that rhGH could promote intestinal mucosal epithelial cell proliferation and protein synthesis in humans.

Innovations and breakthroughs

Few studies have made direct observations of the effects of rhGH on intestinal mucosa in humans. In this study, the effects of rhGH on intestinal mucosa proliferation were directly observed. Intestinal mucosal biopsies were performed by endoscopy through enterocutaneous fistula.

Applications

This study suggests that rhGH may reasonably be applied in a clinical setting.

Peer review

Although this is a very interesting study, it is just a preliminary observation. It should be verified in the future. Authors should comment on possible adverse effects of this drug.

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Johanson-Blizzard syndrome with mild phenotypic features confirmed by *UBR1* gene testing

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Abstract

Johanson-Blizzard syndrome (JBS) is a rare autosomal recessive condition associated with exocrine pancreatic insufficiency, and is characterized by hypoplastic nasal alae, mental retardation, sensorineural hearing loss, short stature, scalp defects, dental abnormalities and abnormal hair patterns. Growth hormone deficiency, hypopituitarism, and impaired glucagon secretion response to insulin-induced hypoglycemia have been reported. Congenital heart defects have also been described in this condition. Mental retardation is typically moderate to severe in patients with JBS; however, normal intelligence can occur. In the pancreas, there is a selective defect of acinar tissue, whereas the islets of Langerhans and ducts are preserved. Diabetes has been reported in older children, suggesting the progressive nature of pancreatic disease. The molecular basis of JBS has recently been mapped to chromosome 15q15-q21 with identified mutations in the *UBR1* gene. We report the case of a 7-year-old female with pancreatic insufficiency and mild phenotypic features, in whom the diagnosis of JBS was established using recently described molecular testing for the *UBR1* gene.

INTRODUCTION

Johanson-Blizzard syndrome (JBS) is a rare autosomal recessive multisystem disorder in which the most characteristic feature is exocrine pancreatic insufficiency. Other common abnormalities include an abnormal facial appearance with a small beak-like nose, dental abnormalities, sensorineural hearing loss, midline scalp defects, hypothyroidism, genitourinary abnormalities, varying degrees of mental retardation, and growth failure^[1,2]. In 2005 the disease-associated locus in individuals with this syndrome was mapped to chromosome 15q15-21 with identified mutations in the gene *UBR1* encoding a ubiquitin ligase of the N-end rule pathway^[3]. We report the case of a 7-year-old patient recently diagnosed with JBS, confirmed by genetic testing, who has been followed for longstanding pancreatic insufficiency of unknown etiology, but with only mild phenotypic features of JBS, mild sensorineural hearing loss, and who is of normal intelligence.

CASE REPORT

The patient is a 7-year-old girl who was initially evaluated at 18 mo of age for a history of growth failure and increased stool frequency. She was a term infant, birth weight 3230 g, born to non-consanguineous parents. She was initially breast-fed and transitioned to a soy-based formula at 3 mo of age and lactose-free milk at 1 year. She tolerated the introduction of solid foods at 4 mo of age. She had a history of 3-5 large bulky stools per day that contained partially undigested food and were described as being occasionally oily. Her growth was below the

Table 1 Initial evaluation

Complete blood count
Hemoglobin 11.5 g/dL (normal, 10.5-13.5)
WBC 7420/ μ L (normal, 5.5-15.5)
ANC 1460/ μ L
Otherwise unremarkable
Complete metabolic panel
Total protein 6.6 g/dL (normal, 6-8.4)
Albumin 4.1 g/dL (normal, 3.5-5)
Alk phos 369 U/L (normal, 80-340)
Otherwise unremarkable
Lipase 8 U/L (normal, 12-70)
Amylase 49 U/L (normal, 0-137)
PT 11.6 s
Vitamin A and D levels were normal
Vitamin E 0.1 mg/dL (normal, 0.5-2)
Celiac antibody testing negative
Serum trypsinogen < 1.2 ng/mL (normal, 10-57)
TSH and free T4 were normal
Stool ova and parasites negative
Seventy-two fecal fat
Fecal fat quant 10.6 g/24 h (normal, < 7)
Coefficient of fat absorption 83%
Stool chymotrypsin < 3 U/10 g (normal, > 9)

Sweat test: 45 mmol/L (normal, 8-45); CFTR gene mutation analysis: No known deleterious mutations; Shwachman-Diamond gene analysis: Negative.

3rd percentile for weight and height. She was otherwise healthy, with the exception of having five ear infections between the ages of 6 and 18 mo, which ultimately required pressure-equalizing tube placement. Her developmental history was normal, other than starting to walk independently at 18 mo of age. Family history was negative for cystic fibrosis, celiac disease, chronic diarrhea and growth failure. Her physical examination was remarkable for a weight of 9.3 kg (7% ile) and a height of 77.6 cm (< 5% ile). Her initial laboratory evaluation is shown in Table 1. She had evidence of significant fat malabsorption; however, testing was negative for celiac disease, cystic fibrosis, and Shwachman-Diamond syndrome. Based on the presumed diagnosis of pancreatic insufficiency, she was started on pancreatic enzyme replacement and fat soluble vitamin supplementation. She gained weight and grew along the 5%-10% ile for height and weight on this regimen. She was otherwise healthy and did not have recurrent or frequent infections. Her development was normal.

At 5 years of age, she failed her routine kindergarten hearing screen. Her parents had previously not noted any problem with her hearing or speech. An audiology evaluation was abnormal demonstrating a mild-to-moderate bilateral asymmetric sensorineural hearing loss, greater on the left than the right. She was referred for genetic evaluation given the known association between JBS and sensorineural hearing loss with her history of pancreatic insufficiency. At that time, mild phenotypic features of JBS were identified, including an abnormal hair pattern, hypoplasia of the nasal alae, small teeth and a narrow upper lip (Figure 1). A computed tomography (CT) scan of her abdomen demonstrated complete fatty replacement of the pancreas with no visualized



Figure 1 Phenotypic features of JBS in our patient: abnormal hair pattern, nasal alae hypoplasia, small teeth and narrow upper lip (with permission from parents).



Figure 2 CT scan of the abdomen demonstrating fatty replacement of the pancreas (arrow).

gland residing in the pancreatic bed (Figure 2). Renal ultrasound to evaluate genitourinary abnormalities was negative.

Research testing for mutations in *UBR1* revealed two novel mutations that molecularly confirmed the diagnosis of JBS, IVS1+5G>C (c.81+G>C) a splice site mutation (paternally inherited, father had a mosaic mutation present in only a subset of cells) and exon 17, c.1979_1981delTTG (p.V660del, which is a deletion of a highly conserved valine that was maternally inherited (Figure 3). While abnormal splicing at the splice donor site of exon 1 is predicted to lead to no expression of a functional protein (functional null allele), the maternally inherited deletion may represent a hypomorphic mutation, conferring partial residual function. Indeed, analysis of mRNA from lymphoblastoid cells from the patient by RT-PCR and sequencing indicated that mRNA from the allele with the splice site mutation underwent early degradation (Figure 3). However, we cannot exclude the possibility that some production of a functional UBR1 protein may also result from a low level of normal splicing despite the splice donor mutation at position +5. Our patient has continued to do well on pancreatic enzyme replacement and is being followed by otolaryngology for her hearing loss. She was referred to dental services for her tooth abnormalities.

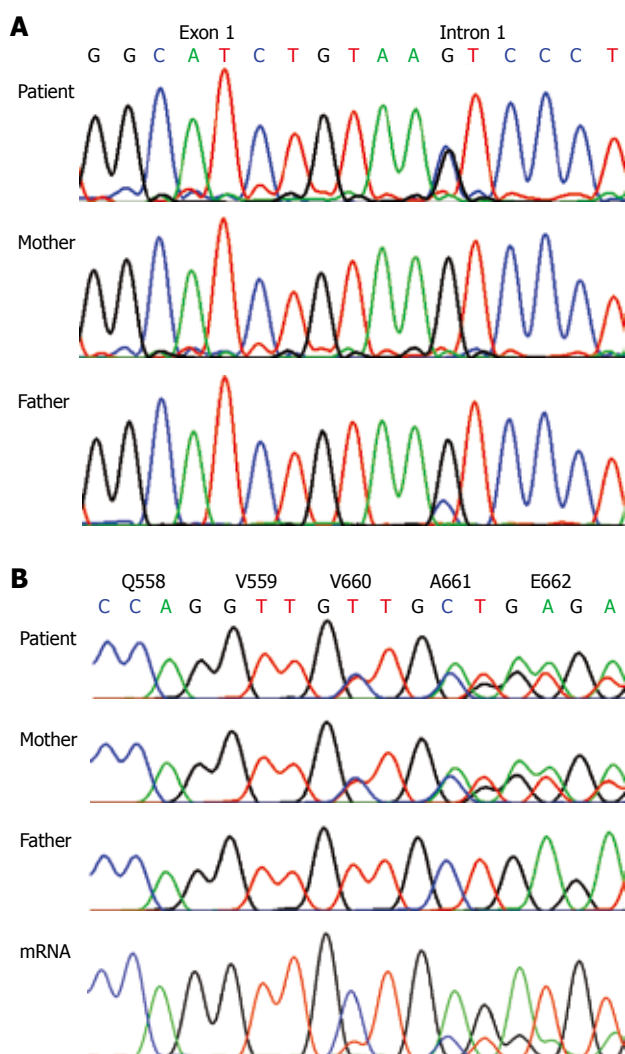


Figure 3 Diagram of the *UBR1* gene mutations in our patient and her parents. A: Exon 1-intron 1 transition showing a heterozygous nucleotide exchange at position +5 in the patient, IVS1+5G>C (c.81+G>C). Note the small peak in the father, indicating that he is mosaic for this mutation. B: Section of exon 17 showing a heterozygous 3 bp deletion in the patient and her mother, c.1978_1981delTTG (p.V660del), predicting the deletion of a highly conserved valine. In mRNA from lymphoblastoid cells from the patient the deletion is the predominant allele, indicating that mRNA from the allele with the splice site mutation is largely degraded.

DISCUSSION

JBS is a rare autosomal recessive multisystem disorder. The most prominent feature of this syndrome is exocrine pancreatic insufficiency. Other abnormalities include a characteristic facial appearance with a small beak-like nose (secondary to aplasia or hypoplasia of the nasal alae), long and narrow upper lip, small pointed chin, abnormalities of both deciduous and permanent teeth, sparse coarse hair/midline scalp defects, short stature in > 80%, hypothyroidism in 40%, sensorineural hearing loss in > 80%, mental retardation in 77%, imperforate anus in 39%, and genitourinary abnormalities in 38%^[4]. Growth hormone deficiency, hypopituitarism, and impaired glucagon secretion response to insulin-induced hypoglycemia have been reported^[5-7]. Congenital heart defects including atrial septal defect, ventricular septal defect, and dextrocardia with transposition of the great

vessels have also been described in this condition^[8]. Mental retardation is typically moderate to severe in patients with JBS, however, normal intelligence can occur^[9]. Growth failure in patients with JBS typically begins in the intrauterine period and continues throughout childhood. Pancreatic hypoplasia with resultant exocrine insufficiency and malabsorption is thought to be responsible. In the pancreas of patients with this condition there is a selective defect of acinar tissue, whereas the islets of Langerhans and ducts are preserved^[10,11]. This results in an almost complete absence of zymogens from duodenal juice, whereas bicarbonate secretion is much less impaired^[10]. Diabetes has been reported in older children, suggesting the progressive nature of pancreatic disease^[12,13].

The molecular basis of JBS has recently been mapped to chromosome 15q15-q21 with identified mutations in the *UBR1* gene^[3,4]. *UBR1* expression is highest in predominantly skeletal muscle and pancreatic acinar cells. *UBR1* encodes one of several E3 ubiquitin ligases of the N-end rule pathway, an ubiquitin-dependent proteolytic pathway. Ubiquitylation and subsequent degradation of proteins at the proteasome is the universal mechanism for regulated protein degradation and the control of many intracellular protein levels^[14-16]. *UBR1* is considered to play a critical role in the development and maintenance of acinar cells. In patients with JBS, destruction of acinar tissue which may begin *in utero* results in the development of exocrine pancreatic insufficiency and fatty replacement of the pancreas. Since the initial description of JBS in 1971, more than 60 cases have been reported^[4]. The majority of these reports include children with significant pancreatic insufficiency, markedly abnormal facial features and moderate to severe mental retardation.

Our patient presented with pancreatic insufficiency and initially unrecognized mild phenotypic features of JBS. This diagnosis was only suspected when she failed a routine screening hearing test, without prior suspicion of hearing loss. In contrast to previous findings of biallelic *UBR1* mutations predicting complete loss of function in the majority of patients with JBS^[3], in our patient, the maternally inherited deletion is thought to be a hypomorphic mutation conferring partial residual function and explaining the more subtle phenotype. This is the first evidence for genotype-phenotype correlation in JBS. The purpose of this report is to highlight the broader spectrum of this syndrome which may have been previously unrecognized prior to the availability of specialized genetic testing. Once the diagnosis of JBS is established, patients with this condition need to be screened for renal anomalies, referred for dental evaluation, monitored for the development of hypothyroidism and diabetes and provided with appropriate genetic counseling.

JBS is a rare cause of pancreatic insufficiency, usually associated with typical phenotypic features. The genetic basis for this syndrome has been recently identified, and is related to *UBR1* deficiency which leads to perturbation of pancreatic acinar cells as well as other

organs. Gastroenterologists should be aware of the availability of genetic testing for JBS. Recognition of more subtle presentations of this syndrome may help to identify other patients with this autosomal recessive condition, previously thought to have idiopathic pancreatic insufficiency.

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Endoscopic Ultrasound-guided drainage of an abdominal fluid collection following Whipple's resection

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INTRODUCTION

Drainage of post-operative abdominal fluid collections is generally carried out percutaneously under radiological guidance. However, some of these collections may be inaccessible to percutaneous drainage because of their location and surrounding vital structures.

We report a case where a post-operative fluid collection which developed after a Whipple's resection was successfully drained endoscopically under endoscopic ultrasound (EUS) guidance. Although widely used in pancreatitis, this is the first report of the use of this technique in a post-operative setting.

CASE REPORT

A 45 year old lady underwent Whipple's resection for cholangiocarcinoma of the distal common bile duct. Pancreatico-jejunostomy and hepatico-jejunostomy were performed on to a 70 cm Roux-en-Y jejunal loop and a gastrojejunostomy was fashioned using the end of the main jejunal segment continuous with the rest of the intestine.

Her initial recovery was uneventful but on the 9th post-operative day she developed pyrexia and vomiting suggestive of gastric outlet obstruction. A contrast-enhanced computerized tomogram (CT) showed a deep-seated fluid collection measuring 5.1 × 3.3 cm which was compressing and stretching the efferent jejunal loop distal to the gastrojejunostomy (Figure 1). Percutaneous drainage was deemed unsafe due to the surrounding bowel loops and blood vessels. However, its proximity to the gastrojejunostomy made it accessible endoscopically.

An EUS-endoscope (GF-UCT240, Olympus, UK) was passed through the gastrojejunostomy and the sero-sanguinous collection was aspirated to dryness under EUS guidance using a 19-gauge Echotip-Ultra needle (Wilson-Cook, Ireland) (Figure 2). The amylase level in the aspirate was normal and all the cultures were sterile. Following aspiration of the fluid collection the

Abstract

Percutaneous aspiration and drainage of post-operative abdominal fluid collections is a well established standard technique. However, some fluid collections are not amenable to percutaneous drainage either due to location or the presence of surrounding visceral structures. Endoscopic Ultrasound (EUS) has been widely used for the drainage of pancreatitis-related abdominal fluid collections. However, there are no reports on the use of this technique in the post-operative setting. We report a case where the EUS-guided technique was used to drain a percutaneously inaccessible post-operative collection which had developed after Whipple's resection.

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Key words: Endoscopic ultrasound; Pancreatectomy; Whipple's resection

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Figure 1 Contrast-enhanced CT showing the fluid collection (black arrow) compressing the efferent jejunal loop (white arrow) just distal to the gastrojejunostomy.



Figure 2 EUS showing the fluid collection (black arrow) with the needle in position for aspiration (white arrow).

vomiting and pyrexia settled, and the patient was able to recommence oral intake. There were no complications related to this procedure and she was discharged from hospital after three days. She was asymptomatic at her routine surgical outpatient review 6 wk later.

DISCUSSION

Abdominal fluid collections are common after Whipple's resection and are frequently situated in the pancreatic bed. They may be related to an anastomotic leak (generally pancreatic) and often cause mechanical and/or infective complications. These collections are generally deep-seated and are surrounded by major blood vessels.

This combined with altered post-surgical anatomy (presence of Roux loops *etc*) often makes a direct percutaneous route for drainage impossible to find. In this patient, it was important to exclude a pancreatic leak and to resolve the gastric outlet obstruction. In such cases, a laparotomy would normally be necessary in the absence of a safe percutaneous option.

In contrast, endoscopic transmural drainage or aspiration with EUS guidance has greatly reduced the need for surgery in pancreatitis-related fluid collections and has now become a standard procedure for this condition in our unit. The safety of this technique and high success rates have been widely reported^[1-3]. This experience prompted us to use the EUS-guided technique as an alternative route for drainage of this post-operative fluid collection where percutaneous approach was deemed unsafe.

In a patient with a visceral fluid collection, the feasibility of EUS-guided drainage should first be assessed with dual contrast-enhanced CT. Close proximity to the stomach or jejunum provides an easy endoscopic access to these collections. The technique of EUS-guided aspiration is well established^[1-3].

We anticipate that the main role of EUS-guided aspiration of collections in the post-operative situation would be to allow much-needed sampling of fluid for microbiological and biochemical analysis. In patients with sepsis or mechanical complications such as obstruction secondary to collections, this technique would also be therapeutic. In stable patients this could eliminate the need for a laparotomy which would otherwise carry a much higher risk of morbidity and mortality. Thus, we conclude that EUS-guided aspiration/drainage should be considered in patients with otherwise inaccessible post-operative fluid collections.

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Complete hepatocellular carcinoma necrosis following sequential porto-arterial embolization

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Zalinski S, Scatton O, Randone B, Vignaux O, Dousset B. Complete hepatocellular carcinoma necrosis following sequential porto-arterial embolization. *World J Gastroenterol* 2008; 14(44): 6869-6872 Available from: URL: <http://www.wjg-net.com/1007-9327/14/6869.asp> DOI: <http://dx.doi.org/10.3748/wjg.14.6869>

Abstract

Most patients with hepatocellular carcinoma (HCC) are not eligible for curative treatment, which is resection or transplantation. Two recent series have emphasized the potential benefits of preoperative arterio-portal embolization prior to surgical resection of such tumours. This preoperative strategy offers a better disease free survival rate and a higher rate of total tumor necrosis. In case of non resectable HCC it is now widely accepted that transarterial chemoembolization (TACE) leads to a better survival when compared to conservative treatment. Thus, the question remains whether combined portal vein embolization (PVE) may enhance the proven efficiency of TACE in patients with unresectable HCC. We herein report the case of a 56-year-old cirrhotic woman with a voluminous HCC unsuitable for surgical resection. Yet, complete tumour necrosis and prolonged survival could be achieved after a combined porto-arterial embolization. This case emphasizes the potential synergistic effect of a combined arterio-portal embolization and the hypothetical survival benefit of such a procedure, in selected patients, with HCC not suitable for surgery or local ablative therapy.

INTRODUCTION

Despite a close observation of patients with liver cirrhosis, hepatocellular carcinoma (HCC) is often diagnosed at an advanced stage where no optimal treatment has been established^[1-3] only few patients (20%-25%) will benefit of resection or liver transplantation^[4], only chance to improve life expectancy. Actually, 80% to 90%^[5] of HCC develop in cirrhotic patients with impaired liver function, limiting the possibility of safe major liver resections. If liver resection has shown a survival benefit, in case of cirrhosis, it's a risky procedure with a high prevalence of postoperative liver failure and should not be performed if the future liver remnant (FLR) is estimated to be less than 40%^[6]. In those patients who are not suitable for surgery, treatment is palliative and survival is poor and correlated to TNM stage (TNM classification of primary liver cancer by the International Hepato-Pancreato-Biliary Association^[7]) and liver damage severity^[8]. Despite previous inconclusive randomized controlled trials comparing transarterial chemoembolization (TACE) to conservative treatment in unresectable HCC^[9-11], Liovet *et al*^[12] ultimately demonstrated that TACE led to an increased survival in selected patients with preserved liver function. TACE is now widely accepted as the procedure of choice in selected patients who are not eligible for

resection or local ablative therapy. However, the question remains whether combined portal embolization may enhance the proven efficiency of TACE in patients with unresectable hepatocellular carcinoma (HCC).

We herein report the case of a cirrhotic patient with advanced HCC in whom complete tumour necrosis and prolonged survival were observed following a combined porto-arterial embolization.

CASE REPORT

A 56-year-old woman with alcoholic cirrhosis, Child-Pugh A6 presented with an 80 mm HCC stage III. The tumor developed in the right hepatic lobe, impinging on the median hepatic vein and in contact with the right glissonian pedicle (Figure 1A). There were two satellite nodules but the left hepatic lobe was free of tumor. The right portal vein was patent. The patient had stopped alcohol intake for 3 mo before admission. There was no past history of encephalopathy, ascites or upper gastrointestinal bleeding despite stage I oesophageal varices. The clinical examination was normal. Liver biochemistry showed: A 74% prothrombin time, normal albumin level, bilirubin 50 $\mu\text{mol/L}$ ($N < 17$), ASAT/ALAT: 59/66 UI/L ($N < 40$), gamma glutamyl transferase: 204 UI/L ($N < 140$), alkaline phosphatase: 105 UI/L ($N < 80$), platelet count was 126 000/ mm^3 . Alpha-foeto protein level was 108 $\mu\text{g/L}$ ($N < 5$). The diagnosis of established alcoholic cirrhosis was confirmed by a percutaneous liver biopsy. As the surgical strategy was a right hepatectomy removing the median hepatic vein and the patient underwent a right portal vein embolization (PVE) prior to surgery (Figure 2A and B). After 4 wk the left lobe had gained 40%. During surgery, intra-abdominal exploration revealed moderate to severe portal hypertension with an enlarged spleen, mild ascites and dilated splanchnic veins. The liver appeared cirrhotic with regeneration nodules. Intraoperative ultrasound confirmed an 8 cm HCC, mainly involving segment V and VIII, invading the median hepatic vein and close to the right glissonian pedicle. Peroperative observation precluded liver resection and separate biopsy of both tumor and liver parenchyma was done before abdominal closure. In view of previous right PVE, intra-arterial chemoembolization was thought to be unsafe and a supportive medical care was decided. During follow-up, a 20 mm intra-tumoral aneurysm of right arterial branch was diagnosed on computer tomography (CT) scan (Figure 1B), most probably related to an arterial trauma during intraoperative tumor biopsy. A supra-selective arterial embolization with coils was then undertaken with complete obliteration of the arterial aneurysm on control angiography (Figure 3A and B). A control CT scan performed 3 mo later showed complete necrosis of the tumor (Figure 1C) as suggested by return of alpha-foeto protein to normal value (6.8 $\mu\text{g/L}$). Disease-free survival lasted for two years. Multiple intra-hepatic and bone recurrence was diagnosed on progressive increase of alpha foeto-protein level. The patient ultimately died after a follow-up of three years.

DISCUSSION

In up to 90%, HCC develop in cirrhotic patients whose impaired liver function precludes major liver resections if FLR is less than 40%^[6]. First reported by Makuuchi *et al*^[13], PVE can be performed safely^[14] in order to induce a homolateral liver parenchyma atrophy and a hypertrophy of the FLR, allowing resection in patients with large tumors or abnormal liver function^[15]. Yet, this technique was initially described for patients with Klatskin tumors^[13] and its' application to cirrhotic patients with HCC is debated by some authors who rather recommend a preoperative combined arterio-portal embolization^[16]. HCC being hypervascular tumours mainly fed by an arterial blood flow, cessation of the portal's flow leads to a compensatory increased flow in the corresponding arterial territory^[17] that may cause the tumor progress.

Recently, Ogata *et al*^[18] have reported in a controlled trial the feasibility and efficacy of a sequential arterio-portal embolization, TACE followed by PVE after a 3 wk delay, before major liver resection in cirrhotic patients with HCC. When compared to PVE alone, this procedure offers a significantly higher rate of complete tumor necrosis (83% *vs* 5%, $P < 0.001$), a higher 5-year disease-free survival rate (37% *vs* 19%, $P = 0.041$) with a similar rate of morbidity. In this report the authors confirmed that complete tumor necrosis can be obtained by its complete blood flow privation (arterial and portal) and highlight the potential benefit of this sequence in term of prognosis. Moreover, in their report, the Beaujon's group^[18] suggests that sequential embolization could effectively be an appropriate treatment itself in patients in whom surgery is precluded due to a poor degree of liver hypertrophy.

In case of unresectable HCC, efficacy of TACE is now admitted^[19,20] with a benefit in survival when compared to conservative treatment. Yet, TACE alone leads to around 50% of complete tumour necrosis^[21-23] whereas this rate is over 80% after sequential arterio-portal embolization and this may have an impact on survival curves^[16,18]. Aoki *et al*^[16] obtained a necrosis rate superior to 70% in 12/17 (71%) whereas Yamakada *et al*^[24] observed a complete tumoral necrosis in 7/9 (78%) of the resected specimen after a sequential arterio-portal procedure with 1, 3 and 5 years survival rates of 87%, 72% and 51%, respectively.

Even though portal vein thrombosis, which is a frequent complication of HCC, is considered to be an absolute contraindication to TACE, due to increased risk of post procedure liver failure or infarction, efficacy and safety of TACE in such cases have been reported^[25] in selected patients. Taken together, these observations suggest a good tolerance of liver parenchyma to ischemia when interval between portal and arterial occlusion is delayed^[18]. Ogata *et al*^[18] who have suggested a minimum of 3 wk between both procedures had lesser morbidity and aminotransferase levels as compared to Aoki *et al*^[16] who performed both embolizations within a period of 7 d.

In the case herein reported, although our patient

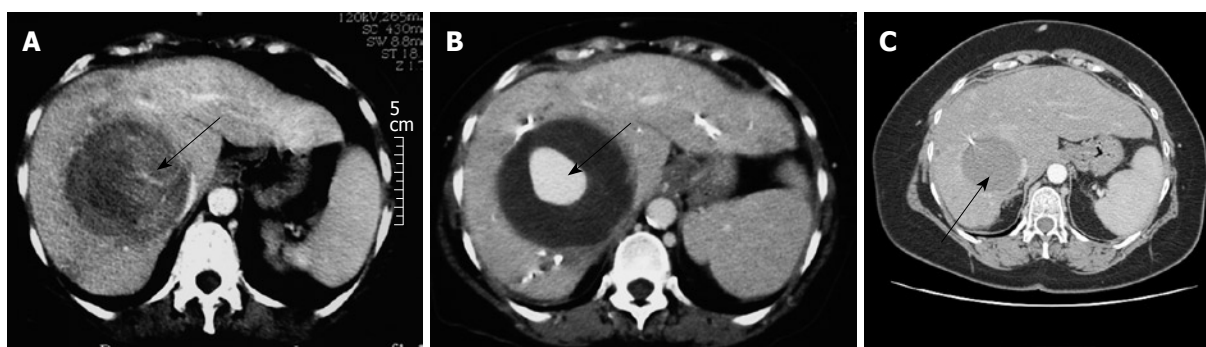


Figure 1 Enhanced CT scan. A: Showing an eighty millimetre HCC (black arrow) developed in the right hepatic lobe, driving back the median hepatic vein, in contact with the right glissonian pedicle; B: Showing an arterial aneurysm (black arrow) due to the main tumour artery traumatism during biopsy; C: Showing complete tumour necrosis after combined portoarterial embolization (black arrow).

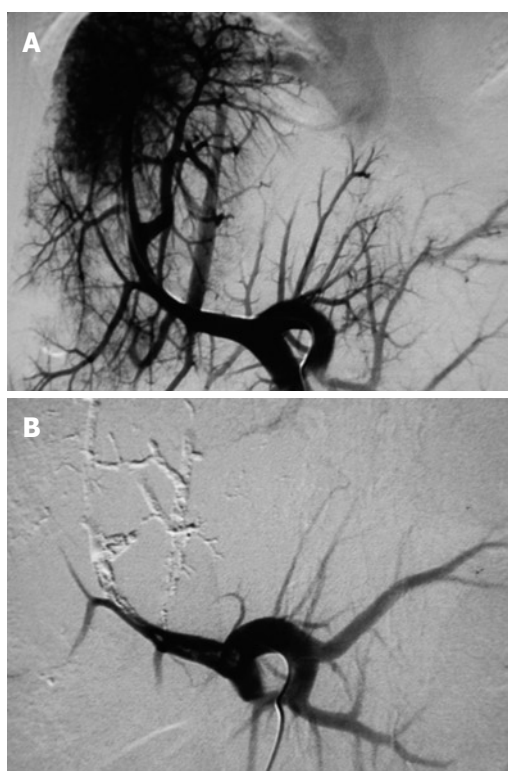


Figure 2 Portography. A: Prior to embolization; B: After embolization.

was planned to have a major liver resection we did not perform a preoperative TACE before PVE, which is now systematic in our department. In this particular case, arterial embolization was performed after portal embolization and was indicated to treat a traumatic arterial aneurysm following a peroperative fine needle biopsy. Yet, it was well tolerated, had no consequence on liver function and led to a complete tumor necrosis with a prolonged survival. We agree that a porto-arterial sequence is unusual and do not recommend it that way, but this case fully illustrates the synergy of a combined embolization in term of tumor necrosis. We rather recommend a sequential arterio-portal sequence combining TACE and PVE as previously described^[18], with a 3 wk delay between both procedures. In this view, we assume that tolerance is related to the sequence and its timing whereas efficacy is related to both, arterial and

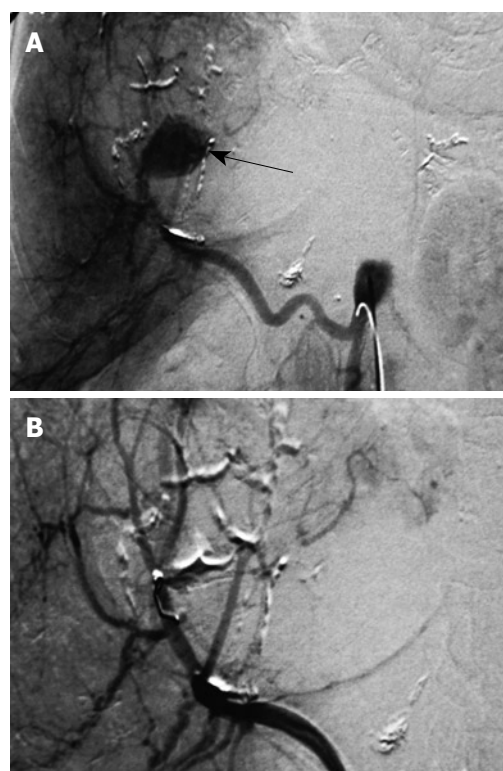


Figure 3 Arteriography. A: The arterial aneurysm developed on the HCC main feeding artery (black arrow), prior to embolization with coils; B: After complete arterial occlusion with coils.

portal, HCC vascular exclusion.

Our report aims at giving further support to the combination of TACE and portal embolization in the treatment of voluminous HCC that cannot be treated by surgery or alternative therapy such as radiofrequency as previously hypothesized by others^[18]. We assume that cirrhotic liver parenchyma has a relatively good tolerance to arterial and portal ischemia when the interval between both vascular occlusions is delayed (at least 3 wk). Proven efficacy of TACE might be enhanced by a combined sequential PVE. Patients With large HCC, not suitable for surgery or local ablative therapy, could effectively be treated with combined arterio-portal embolization with a limited morbidity and a likely benefit in survival.

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Cystic lymphangioma of the pancreas

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Abstract

Lymphangioma of the pancreas is an extremely rare benign tumour of lymphatic origin, with fewer than 60 published cases. Histologically, it is polycystic, with the cysts separated by thin septa and lined with endothelial cells. Though congenital, it can affect all age groups, and occurs more frequently in females. Patients usually present with epigastric pain and an associated palpable mass. Complete excision is curative, even though, depending on the tumour location, surgery may be simple or involve extensive pancreatic resection and anastomoses. The authors present a 49-year-old woman in whom a polycystic septated mass, 35 mm x 35 mm in size, was discovered by ultrasonography (US) in the body of the pancreas during investigations for epigastric pain and nausea. At surgery, a well circumscribed polycystic tumor was completely excised, with preservation of the pancreatic duct. The postoperative recovery was uneventful. Histology confirmed a microcystic lymphangioma of the pancreas. Immunohistochemistry showed cystic endothelial cells reactivity to factor VIII-RA (++), CD31 (+++) and CD34 (-). Postoperatively, abdominal pain disappeared and the patient remained symptomfree for 12 mo until now. Although extremely rare, lymphangioma of the pancreas should be taken

into consideration as a differential diagnosis of a pancreatic cystic lesion, especially in women.

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Key words: Pancreas; Cystic lymphangioma; Local surgical excision

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INTRODUCTION

Lymphangiomas are rare benign cystic tumours that probably occur as a result of congenital malformations of the lymphatics leading to the obstruction of local lymph flow and the development of lymphangiectasia. Histopathologically, they are composed of dilated cystic spaces containing proteinaceous eosinophilic fluid, separated by fine septa and lined with endothelial cells^[1]. These tumours present most frequently in childhood^[2] and have an associated broad spectrum of clinical symptoms, depending on the disease location. They are most commonly found in the neck (75%) and the axillae (20%), though a variety of other sites have been described including the mediastinum, pleura, pericardium, groin, bones and the abdomen^[2,3].

Lymphangioma of the pancreas is extremely rare accounting for less than 1% of these tumours^[4], and with only 60 previously reported cases. We present the rare case of an adult with lymphangioma of the pancreas and review the literature.

CASE REPORT

A 49-year-old women presented with increasing upper abdominal pain and nausea in November 2007. She had a past medical history of a uterine myomectomy in 1997, and a hysterectomy and left oophorectomy in 2006. On examination, she was

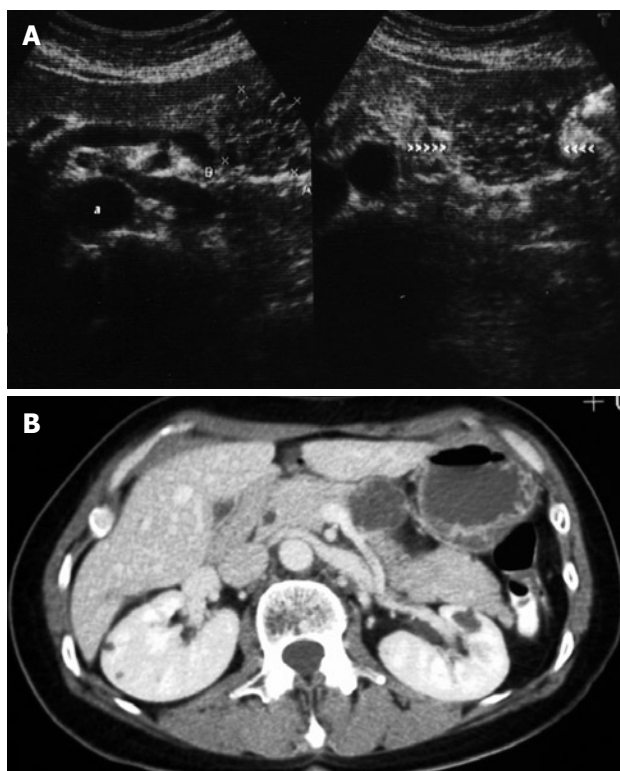


Figure 1 A well-circumscribed 35 mm polycystic lesion in the body of the pancreas, with thin septa within the lesion. A: US scan demonstrating the polycystic tumour of the body of the pancreas; B: CT scan showing the cystic tumour with fine septa.

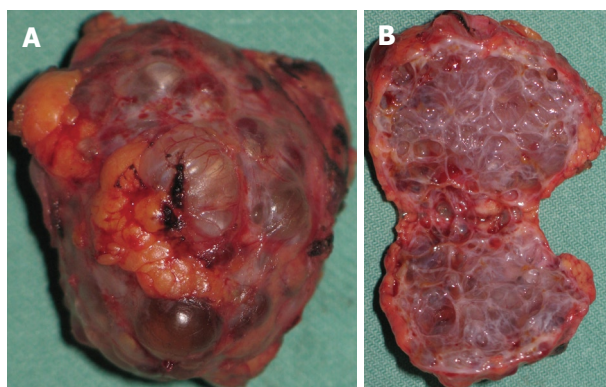


Figure 2 Tumour surrounded by normal pancreatic tissue. A: The excised polycystic mass; B: The tumour after sectioning.

found to be slightly tender at the epigastrium, and laboratory analyses were all within normal limits. An ultrasonography (US) and computer tomography (CT) scan revealed a well-circumscribed 35 mm polycystic lesion in the body of the pancreas, with thin septa within the lesion (Figure 1).

At laparotomy the lesion was found in the lower part of the body of the pancreas, and did not involve the main pancreatic duct. The lesion was completely excised and the main pancreatic duct was preserved. No other pathology was found within the abdomen, and the postoperative recovery was uneventful. Abdominal pain disappeared postoperatively and the patient has been doing well for the following 12 mo until now.

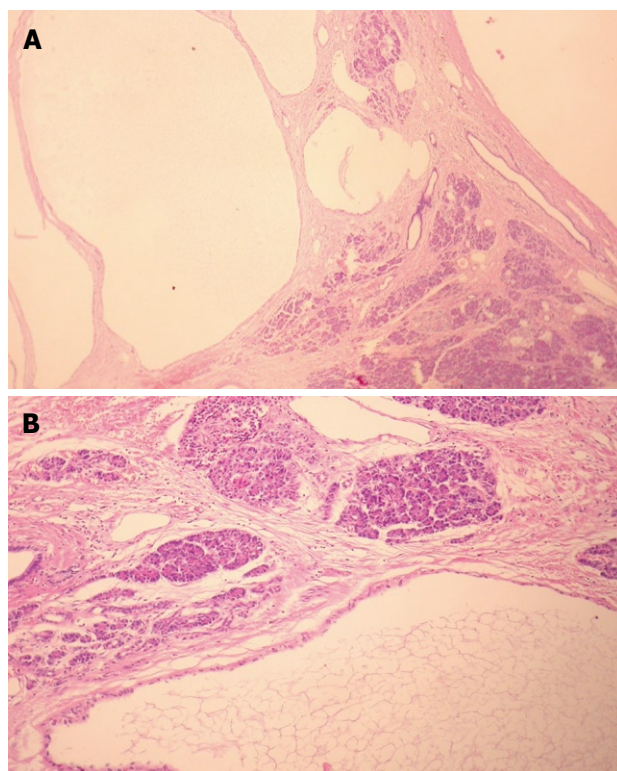


Figure 3 Microscopically all the sections (HE stain). A: Vascular spaces of the pancreatic cystic Lymphangioma containing predominantly clear fluid with few erythrocytes or lymphocytes (x 13); B: The cysts and dilated lymphatics in the surrounding pancreatic tissue are lined with a thin endothelial layer (x 64).

The tumour, measuring 34 mm × 32 mm × 29 mm, had a nodular, gray-blue surface and was surrounded by normal pancreatic tissue (Figure 2A). On sectioning, it had a honeycomb appearance with 1-7 mm polycystic spaces filled with murky haemorrhagic yellowish fluid (Figure 2B). Microscopically, all the sections showed a polycystic structure composed of ectatic lymphatics lined with endothelial cells (Figure 3). The cysts were separated by thin hypocellular septa similar in appearance to the thin capsule surrounding the tumour mass itself. No cell atypia was found. Immunohistochemistry showed immunoreactivity to the factor VIII-R antigen (+++), CD 31 positivity (+++) and CD 34 negativity (-). The final histological diagnosis was of microcystic lymphangioma of the pancreas.

DISCUSSION

Lymphangioma of the pancreas is rare, accounting for less than 1% of lymphangiomas^[4]. It occurs more frequently in females and is often located in the distal pancreas^[5]. The tumour size may vary between 3 and 20 cm in diameter (average 12 cm)^[6]. Patients usually present with abdominal pain^[5] and an associated palpable abdominal mass^[7-9], although an acute abdomen has also been described^[10]. Pancreatitis, weight loss, and laboratory abnormalities are not usual disease manifestations^[1]. US typically shows a polycystic tumour, and calcifications, which are typical for cystadenomas of the pancreas, are very rare^[11]. On CT, the tumour

is a well-circumscribed, encapsulated, water-isodense, polycystic tumour with thin septa, similar in appearance to cystadenomas, which occur far more frequently^[1,12].

Differential diagnoses include pancreatic pseudocysts, mucinous and serous cystadenomas, other congenital cysts and pancreatic ductal carcinoma with cystic degeneration^[1,13,14]. The final diagnosis is histological^[1], with the endothelial cells showing immunohistochemical reactivity to factor VIII/R antigen, CD 31 (+) positivity^[6,8] and CD 34 (-) negativity^[6], as seen in our patient.

A complete surgical excision is curative^[6,8,15], with incomplete excision being the only reason for recurrent disease^[7]. Depending on the tumour location and size, complete excision may involve a simple marginal tumorectomy^[10] or may require larger pancreatic resections with anastomoses.

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CASE REPORT

A patient with unresectable advanced pancreatic cancer achieving long-term survival with Gemcitabine chemotherapy

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Unresectable advanced pancreatic cancer

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Okamoto Y, Maeba T, Kakinoki K, Okano K, Izuishi K, Wakabayashi H, Usuki H, Suzuki Y. A patient with unresectable advanced pancreatic cancer achieving long-term survival with Gemcitabine chemotherapy. *World J Gastroenterol* 2008; 14(43): 6876-6880 Available from: URL: <http://www.wjgnet.com/1007-9327/14/6876.asp> DOI: <http://dx.doi.org/10.3748/wjg.14.6876>

Abstract

A 68-year-old female visited a local clinic with epigastralgia. A routine laboratory test revealed jaundice and liver dysfunction. She was referred to this hospital. Abdominal computed tomography (CT) and endoscopic retrograde cholangio-pancreatography (ERCP) revealed that the density of the entire pancreas had decreased, and showed dilatation of the common bile duct (CBD) and the main pancreatic duct (MPD). Pancreatic cancer was diagnosed by cytological examination analyzing the pancreatic juice obtained by ERCP. When jaundice had decreased the tumor was observed *via* laparotomy. No ascites, liver metastasis, or peritoneal dissemination was observed. The entire pancreas was a hard mass, and a needle biopsy was obtained from the head, body and tail of the pancreas. These biopsies diagnosed a poorly differentiated adenocarcinoma. Hepaticojejunostomy was thus performed, and postoperative progress was good. Chemotherapy with 1000 mg/body per week of gemcitabine was administered beginning 15 d postoperatively. However, the patient suffered relatively severe side effects, and it was necessary to change the dosing schedule of gemcitabine. Abdominal CT revealed a complete response (CR) after 3 treatments. Therefore, weekly chemotherapy was stopped and was changed to monthly administration. To date, for 4 years after chemotherapy, the tumor has not reappeared.

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Key words: Gemcitabine; Long-term survival;

INTRODUCTION

Only 10%-20% of patients with pancreatic cancer present with localized or potentially resectable disease at the time of diagnosis, and the majority of patients are diagnosed at either an unresectable or metastatic stage^[1]. The prognosis of patients with advanced unresectable pancreatic cancer remains very poor. When pancreatic cancer is diagnosed, the disease progresses and curability remains unsatisfactory, especially in stage IVb pancreatic cancer^[2]. The Japanese Pancreatic Cancer Registry reported that the mean survival of patients with stage IVb pancreatic cancer is 6.2 mo^[3], and palliation for the relief of jaundice, duodenal obstruction, or pain is required for these patients. Recently, gemcitabine was developed for the treatment of advanced pancreatic cancer, and current studies have reported improvements in survival as well as clinical benefit in patients^[4]. Burris et al. reported that gemcitabine was more effective than 5-fluorouracil in the alleviation of some disease-related symptoms and improved survival was seen in patients with advanced pancreatic cancer^[5].

This report describes a case of advanced pancreatic cancer which was initially diagnosed during laparotomy as unresectable; however, the patient achieved long-term survival of over 4 years, following a treatment regimen of gemcitabine chemotherapy.

CASE REPORT

A 68-year-old female visited a local clinic complaining of epigastralgia. Routine laboratory tests revealed that she had jaundice (T. bil 6.5 mg/dL) and liver dysfunction

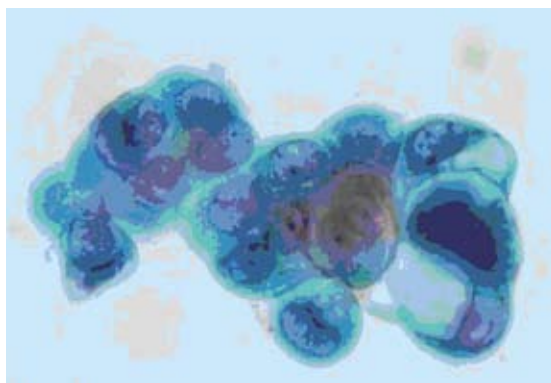


Figure 1 Cytological findings from pancreatic fluid. An adenocarcinoma was indicated.

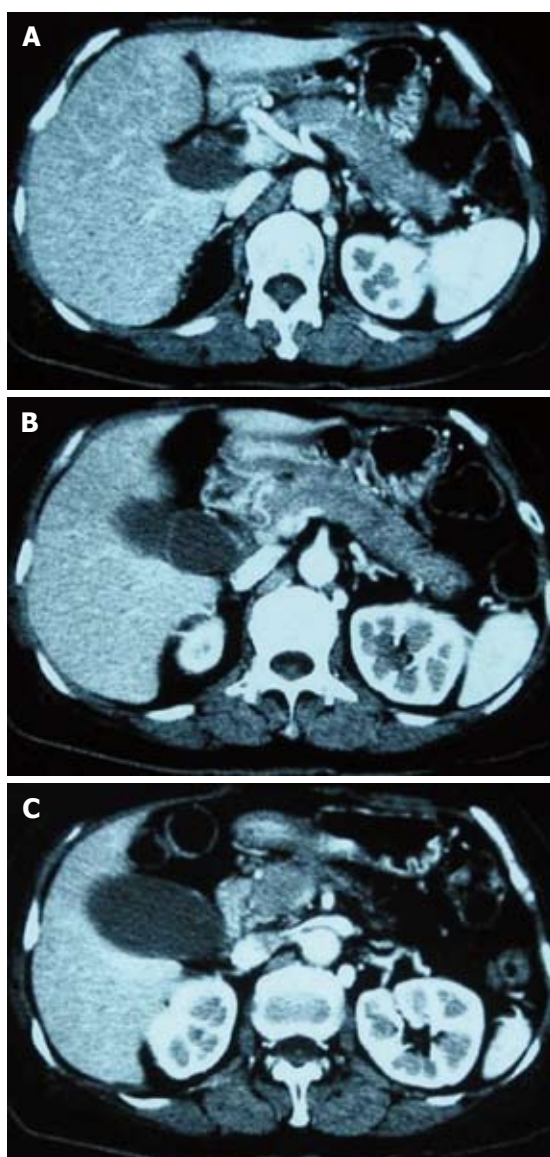


Figure 2 Abdominal CT before surgery. A low density area was observed throughout the entire pancreas, the common bile duct and main pancreatic duct were dilated, the intra-hepatic biliary duct was not dilated. CT: Computed tomography.

(GOT 416 IU/L, GPT 489 IU/L, ALP 2055 IU/L and γ GTP 190 IU/L). Initial laboratory studies included

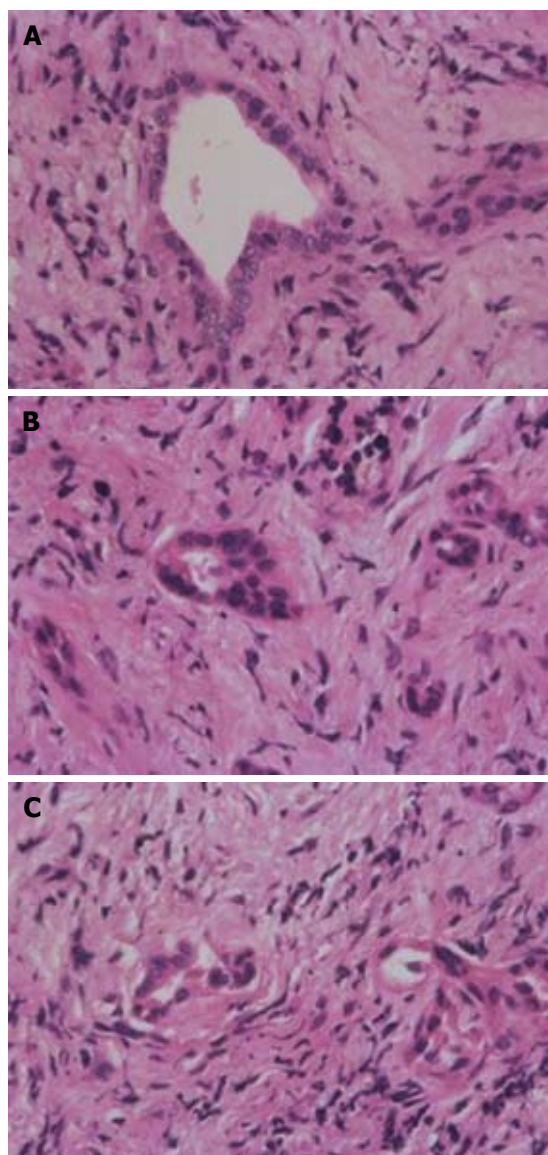


Figure 3 Needle biopsy performed during laparotomy. A: From the head; B: From the body; C: From the tail of the pancreas. All specimens were diagnosed as poorly differentiated adenocarcinoma.

a serum total bilirubin value of 2.6 mg/dL, serum glutamic-oxaloacetic transaminase (GOT) of 416 U/L, serum glutamic-pyruvic transaminase (GPT) of 489 IU/L, alkaline phosphatase (ALP) of 2055 IU/L, and γ -glutamyl transferase (γ GTP) of 190 IU/L. Her CA19-9 was not elevated (27.8 U/mL). She was referred to this hospital. She had a past history of rhabdomyosarcoma of the urinary bladder at the age of 56. On admission, abdominal computed tomography (CT) revealed that the density of the whole pancreas had decreased, and showed dilatation of the common bile duct (CBD) and the main pancreatic duct (MPD). In addition, stricture of the lower CBD was revealed on magnetic resonance cholangio-pancreatography (MRCP) (Figures 1 and 2). The MPD in the pancreas head appeared irregular on endoscopic retrograde cholangio-pancreatography (ERCP). The presence of pancreatic cancer or biliary duct cancer was suspected, because a cytological examination of the pancreatic juice obtained by ERCP

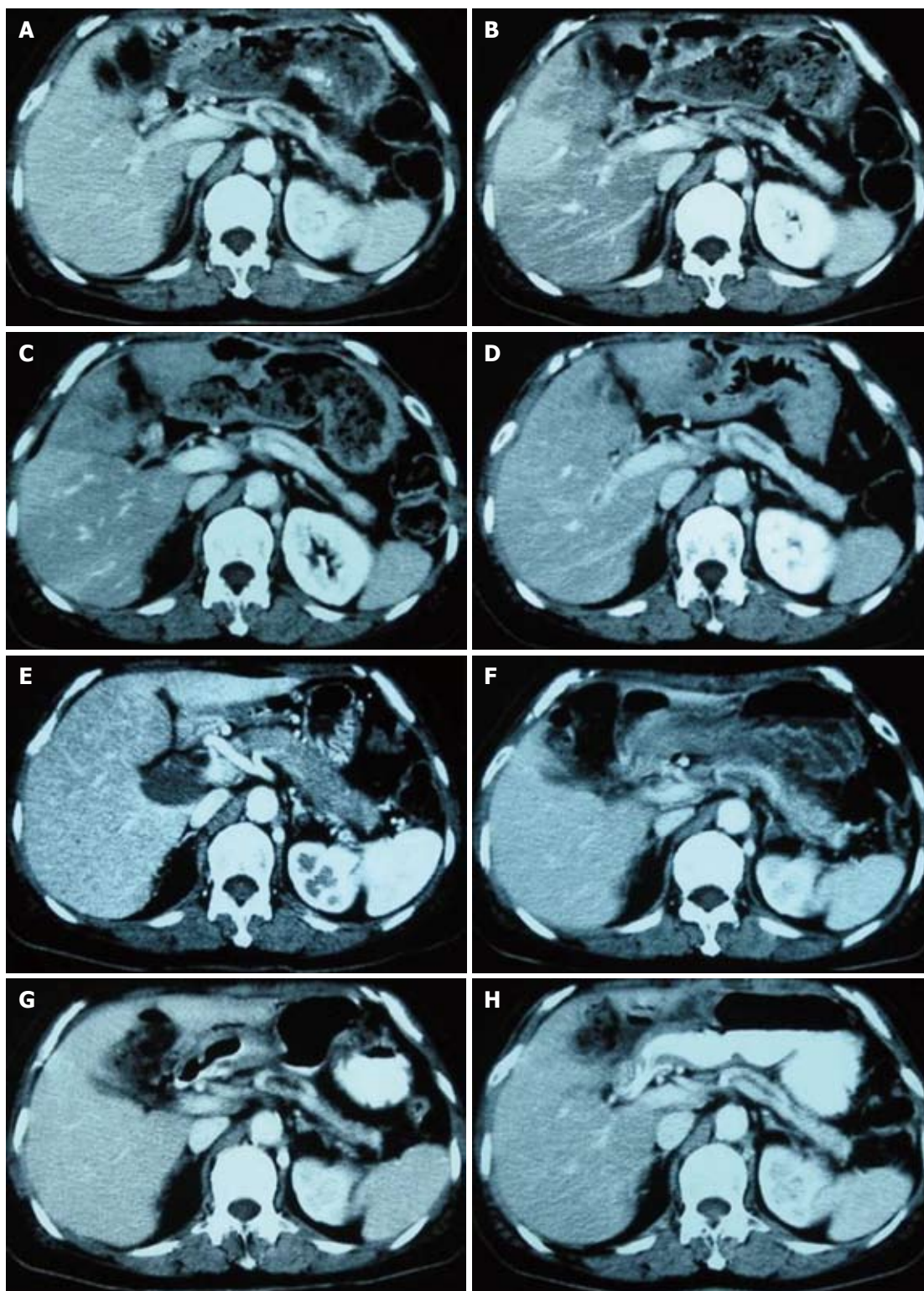


Figure 4 Abdominal CT. A: 12 mo after CTx; B: 16 mo after chemotherapy (CTx); C: 18 mo after CTx; D: 24 mo after CTx; E: preoperative; F: 1 mo after CTx; G: 2 mo after CTx; H: 6 mo after CTx; CT: Computed tomography; CTx: Chemotherapy.

detected adenocarcinoma cells. After amelioration of her jaundice by biliary drainage through percutaneous transhepatic gallbladder drainage (PTGBD), surgical treatment was selected based on a presumptive diagnosis of pancreatic cancer or biliary duct cancer. Laparotomy revealed no liver metastasis or peritoneal dissemination. The entire pancreas was hard, and a needle biopsy

was obtained from the head, body, and tail of the pancreas. Examination of frozen sections revealed a poorly differentiated adenocarcinoma in each specimen (Figure 3). We therefore considered total pancreatectomy, however, a radical resection was considered excessive. Hepaticojejunostomy was performed. The postoperative course was uneventful. On postoperative day 14,

administration of chemotherapy with 1000 mg/body per week of gemcitabine was initiated. However, because the patient experienced adverse effects, such as eyelid swelling, larynx swelling, grade 1 fever and grade 3 leucopenia and neutropenia, the chemotherapy schedule was thus changed to biweekly, with the additional of granulocyte colony stimulating factor (G-CSF) (Figure 4).

After 3 courses of chemotherapy, an abdominal CT scan revealed a complete response (CR), in which the decrease in density of the entire pancreas had disappeared, and dilatation of the MPD had improved. Therefore, weekly chemotherapy was stopped after 3 courses, and changed to monthly chemotherapy with hospitalization. To date, 4 years after the initial chemotherapy, the tumor has not reappeared.

DISCUSSION

The incidence of pancreatic cancer has recently increased worldwide, and the prognosis of patients with this disease remains very poor^[2,6]. Pancreatic cancer is a major cause of cancer-related mortality in Japan and remains one of the most aggressive diseases in the world. In fact, the National Registry of the Japan Pancreas Society reported that only 9.7% of these patients attained 5-year survival^[7]. Early-stage diagnosis of pancreatic cancer is difficult because of the lack of specific early symptoms, and surgery with a curative intent can be performed in only 5%-20% of patients^[2]. When the diagnosis is made, less than 10% of patients will survive for a year, and many require relief from jaundice and the symptoms of gastric outlet obstruction^[2,5,8,9]. The prognosis for unresectable pancreatic cancer thus remains extremely poor.

Gemcitabine was recently developed for the treatment of advanced pancreatic cancer, and current studies have reported an improved survival as well as clinical benefit in patients treated with this agent^[5,10]. Gemcitabine is a prodrug that requires initial intracellular phosphorylation by deoxycytidine kinase, ultimately undergoing phosphorylation to the active gemcitabine triphosphate, a cytotoxic agent that inhibits DNA synthesis.

Today an extensive pancreatic resection may not influence survival, although some studies have recommended an extensive resection for advanced pancreatic cancer^[11,12]. Gemcitabine has been reported to be a strong factor in influencing the survival of patients after resection of pancreatic cancer, and the resection techniques used may not influence a patient's survival when gemcitabine is administered^[9]. Although stage IVb pancreatic cancer without distant metastasis can technically be resected, it normally does not lead to a good recovery and a satisfactory QOL for the patient. As a result, gemcitabine may be the most effective clinically proven treatment for patients with stage IVb pancreatic cancer.

It is interesting that this case achieved a lengthy CR of 4 years after a gemcitabine dose of 800 mg/m² per month. The prognosis of pancreatic cancer is still

poor, despite the findings in this patient who achieved long-term survival following the administration of gemcitabine for progressive pancreatic carcinoma which could not be excised. In addition, such a high success is surprising. Recently, the receptivity of anti-cancer drugs in cancer therapy has been shown to be remarkable, and there may be a specific mechanism which results in the high receptivity of gemcitabine in pancreatic cancer. The publication of further studies on the use of gemcitabine is therefore necessary. In several phase III studies^[5,13-23], objective responses were observed in 4.4%-17.3% of patients treated with gemcitabine alone. Progression-free survival in patients treated with gemcitabine alone was 2-4 mo and median survival was 5.4-7.3 mo for gemcitabine alone. In these studies, patients received either 1000 mg/m² per week of gemcitabine for 3 wk out of every 4 or for 7 wk out of every 8.

Total pancreatectomy results in complete ablation of the exocrine and endocrine pancreatic functions. Despite the administration of insulin and pancreatic enzyme replacement, total pancreatectomy often results in uncontrollable diabetes, as well as persistent diarrhea and steatorrhea, which compromises patients' nutritional status.

In this case, long-term CR and long-term survival were achieved without excision, however, this is a very rare case. The development of gemcitabine has brought about a change in the treatment of pancreatic cancer, but the effect is less than 20%, and the drug has yet to demonstrate satisfactory results. Gemcitabine may be used as second line treatment in patients who have failed first line therapy. Early detection of pancreatic cancer is important for treatment selection, and gemcitabine has been shown to be effective in some pancreatic cancers, as it was in this case. When it is deemed that an aggressive operation would clearly be disadvantageous to a patient, then the patient may undergo conservative surgery followed by appropriate chemotherapy.

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Strangulated small bowel hernia through the port site: A case report

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Abstract

Port site hernia develops through a fascial or peritoneal layer that was inadequate or not repaired. It is a rare complication of laparoscopic surgery which may lead to serious problems. Here, we present a 77-year-old female, diagnosed with a small bowel hernia through a 10-mm port site. We had performed ten cases of laparoscopy-assisted distal gastrectomy before this case. The patient complained of left lower abdominal pain with a palpable mass. Abdominal CT showed an incarcerated small bowel hernia and the patient underwent segmental resection of the strangulated small bowel through a minimally extended port site incision.

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Key words: Port site hernia; Strangulated small bowel; Minimally extended port site incision

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Lee JH, Kim W. Strangulated small bowel hernia through the port site: A case report. *World J Gastroenterol* 2008; 14(44): 6881-6883 Available from: URL: <http://www.wjgnet.com/1007-9327/14/6881.asp> DOI: <http://dx.doi.org/10.3748/wjg.14.6881>

INTRODUCTION

Recently, laparoscopy-assisted gastrectomy (LAG) for early gastric cancer has been accepted as technically and oncologically safe and feasible. During LAG, 10 mm or larger trocars are used for traction of the intra-abdominal organs, and fascial defects created by those trocars are usually closed because of concern over the potential for small bowel hernia. Port site hernia is a rare complication following laparoscopic surgery: however, occurrence of this may be dangerous and, hence, may have a fatal outcome. Therefore, we report the clinical course of our case and prepare a review for the management of port site hernia.

CASE REPORT

A 77-year-old woman was admitted to our hospital for gastric cancer treatment. She had complained of dyspepsia and epigastric discomfort for 20 d: following gastrofiberscopy, early gastric cancer (type I + IIa) was found at the prepyloric antrum. She had no past medical history except well-controlled hypertension, and no abnormalities were found during preoperative evaluation. During laparoscopic surgery, a total of 5 trocars were applied to perform surgical procedures, and all the trocars used were the bladed type. A 10 mm trocar was inserted into the abdominal cavity via the umbilicus to prepare the pneumoperitoneum for electro-laparoscopy. After sufficient inflation of the abdominal cavity, 5 mm and 12 mm trocars were placed into the right side, and 5 mm and 10 mm trocars were inserted into the left side. She underwent LAG with D1 + β lymph node dissection and Billroth-II gastrojejunostomy. The fascial defects at the 10 mm and 12 mm port sites were repaired by 2-0 absorbable vicryl sutures. The patient's recovery was progressing favorably, so we put her on a diet on the 2nd day after surgery, and no abnormalities were detected. On the 7th day after the operation, the patient complained of vague abdominal pain and vomiting, but those symptoms were alleviated by conservative support. The next day, however, the patient developed a fever (38°C) and manifested left lower quadrant abdominal pain with a palpable mass. Emergency ultrasonography and abdominal CT were performed, and a structure that looked like an abscess

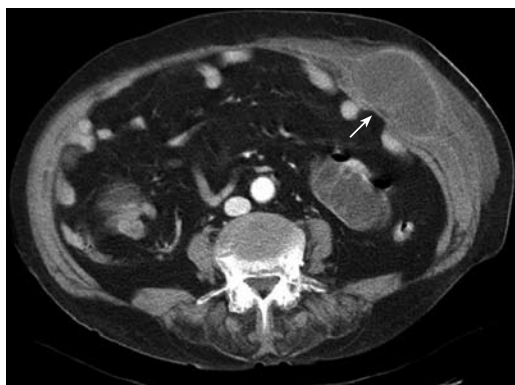


Figure 1 Port site hernia located at the left flank on computed tomography. Arrow indicates hernia.

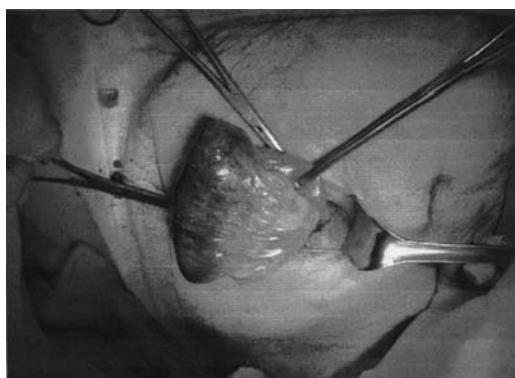


Figure 2 Strangulated small bowel was pulled through the extended port site.

or hernia of the small bowel was detected (Figure 1). We suspected an incarcerated port site or strangulated hernia of the small bowel and performed exploratory surgery. The port site incision that had been made on the left lower quadrant was transversely extended to about 4 cm long. After fascial layer sutures were untied, it was observed that the strangulated small bowel was pushing into a peritoneal defect in the abdominal wall (Figure 2). The length of incarcerated small bowel was about 10 cm long. After that was taken out through an extended incision, we carefully observed its viability and decided to remove a ischemic portion of the small bowel through an extended port site. Then we performed segmental resection of the strangulated small bowel and end-to-end anastomosis. After the operation, there were no specific events, and the patient recovered.

DISCUSSION

Port site hernia is a rare complication following laparoscopic surgery. Tonouchi *et al*^[1] reported that the incidence of port site hernia was 0.65%-2.80% and classified port site hernia into three types. The early-onset type occurs immediately after the operation, the late-onset type develops after several months and the special-type presents as dehiscence of the whole abdominal wall. Relative to the size of the port site,

Kadar *et al*^[2] reported that incidence of port site hernia was 0.23% at a 10 mm port site and 3.1% at a 12 mm port site, and Mayol *et al*^[3] reported umbilical port site hernia with an incidence of 1.6%. Port site hernia may occur at any time during the postoperative course, though our case occurred early during the postoperative course^[4-6]. It is known that a port site of larger than 10 mm in diameter usually causes hernia while a 5 mm port site rarely causes hernia^[5]. Hence, most surgeons routinely repair the fascia of 10 mm or larger port sites to prevent hernia.

In an experimental study of wounds relative to the type of trocar, Kolata *et al*^[7] reported that a non-bladed trocar created smaller peritoneal port site wounds than a conventional pyramidal tip trocar and they hypothesized that a non-bladed type of trocar might reduce the chance of trocar wound hernia. Another study showed that a 10 mm or 12 mm port site created by a non-bladed trocar did not require fascial closure if it was placed in a non-midline position above the arcuate line^[8]. Since the non-bladed trocar split the muscle rather than cutting it, it allowed the oblique muscle to reanneal together more readily. Moreover, this study suggested that misalignment of the fascial defect (created by the non-bladed trocar) aided inhibition of trocar site hernia. However, recently non-bladed trocars have mostly been used to access the port site; hence, many cases occur under this condition and some reports have suggested that herniation through the peritoneal defect might develop even though the fascial defect has been repaired. In our case, an intestinal hernia via peritoneal defect developed below the repaired fascial layer. Therefore, fascial and, if possible, peritoneal repair of port sites are necessary to prevent port site hernia.

Many methods and devices for closure of port sites have been introduced^[9,10], and each of them has its merits and demerits. Generally, it is not difficult to repair port sites that are larger than 10 mm using a small retractor and hemostat unless a patient is severely obese. However, if the port site is less than 5 mm, it will be hard to close and extension of the port site may be necessary for repair. Some devices, such as a Deschamps needle, a suture carrier, and an Endoclose suture device (Tyco Auto Suture International, Inc. Norwalk, CT, USA), may be helpful in repairing fascial and peritoneal defects^[9]. In addition to direct repair of the port site, recently the port plug technique without suturing was introduced. Moreno-Sanz *et al*^[10] reported that inserting a Bioabsorbable Hernia Plug (W.L Gore and Associates Inc, Flagstaff, AZ, USA) to the port site was safe and feasible as a way to prevent port site hernia. Before this unexpected event, trocar sites had been imprudently repaired. After that incident, we made every effort to repair the fascial and peritoneal defects of the port site using an extremely concaved needle and a hemostat as far as possible. After skin and subcutaneous tissue were retracted to opposite sites using small retractors, visualized fascia were clamped using a hemostat and the hemostat was then lifted to allow for detection of

the peritoneum. After the peritoneum was clamped by the hemostat, the two layers were closed separately or together. This technique was similar to the dual-hemostat technique introduced by Spalding *et al.*^[11] and was feasible and simple to perform unless a patient was severely obese.

Clinical courses of port site hernia are varied and depend on the degree of intestinal entry to the defect. Most patients present with vague abdominal pain, nausea, vomiting, a palpable or painful mass around port site, and fever if the bowel is incarcerated. If a patient complains of mild symptoms including nausea, vomiting and vague abdominal pain, early diagnosis may be delayed. Plain radiographs may be useful in some cases; however, plain radiographs only reveal the existence or nonexistence of the bowel obstruction. Therefore, they are limited in diagnosing port site hernia. In the case of Boughey *et al.*^[12], a definite diagnosis was achieved by explorative laparoscopic examination. Abdominal CT may be an effective diagnostic method if a patient complains of the symptoms described above without a palpable mass as it can differentiate adhesion from port site hernia and indicate the location of the port site hernia. Therefore, if atypical symptoms persist with obscure plain radiographs, abdominal CT is helpful in early diagnosis. Exploratory surgery with reduction of the incarcerated bowel is used to treat a port site hernia. According to previous reports, there are a few ways to access the abdominal cavity. One is a laparoscopic approach and another is an open approach through extension of the port site involved or other incision sites. Unless the incarcerated bowel is frankly ischemic, the laparoscopic approach is an acceptable method. In our case, we extended the port site and corrected the disorder because strangulation of the bowel was apparent.

In conclusion, it is necessary to repair the fascial and peritoneal layers to prevent port site hernia. Although port site hernia is rare, surgeons should keep this

possibility in mind. Prompt intervention may reduce unfavorable events if port site hernia is suspected.

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CASE REPORT

Gastric carcinoid tumor in a patient with a past history of gastrointestinal stromal tumor of the stomach

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INTRODUCTION

Gastrointestinal stromal tumors (GIST) are the most frequent mesenchymal tumors in the gastrointestinal tract. They may coexist with other types of cancers with a reported frequency from 4.5% to 33%^[1,2]. The most common location of a GIST associated with other cancers is the stomach^[1], and the most frequently associated tumor type is gastrointestinal adenocarcinoma^[1,2]. Infrequently, other primary cancers have been reported along with GIST, including lymphoma, leukemia, breast cancer, prostate cancer, pancreatic adenocarcinoma, or lung cancer^[1-4]. GIST has been reported to occur simultaneously with ileal carcinoid tumor^[5]. We report a patient with both GIST and carcinoid tumor in the stomach, an association that has not been previously reported.

CASE REPORT

A 65-year-old woman had undergone subtotal segmental gastrectomy for a 5.5 cm GIST in the lower gastric corpus. The diagnosis was based on immunohistochemical staining that was diffusely positive for CD-117 and focally positive for CD-34 and smooth muscle actin; it was negative for desmin. The patient received periodic follow-up gastroscopy. One year after surgery, she was found to have two distinct sessile polypoid lesions in the cardia and lower body of the stomach (Figure 1). The lesion in the cardia, about 2 cm distal to the gastroesophageal junction was a 1.2 cm polyp with a central ulceration. The other lesion was a 0.6 cm mass on the anterior wall near the earlier anastomosis. Immunohistochemical staining of a biopsy specimen of the larger polyp was strongly positive for

Abstract

Gastrointestinal stromal tumor is the most common mesenchymal tumor in the gastrointestinal tract. It may coexist with other type of cancers, and if so, the tumors usually involve the stomach. The most common associated cancers are gastrointestinal carcinomas. We report a 65-year-old woman with a history of gastric gastrointestinal stromal tumor who had undergone subtotal segmental gastrectomy. New polypoid lesions were detected on a follow-up gastroscopy one year later. The lesions were biopsied and found to be carcinoid tumors. There was serum hypergastrinemia, and type 1 gastric carcinoid tumor was diagnosed. A total gastrectomy was performed. Pathologic examination revealed both carcinoid tumors and a recurrent gastrointestinal stromal tumor.

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Key words: Hypergastrinemia; Multiple primary neoplasms; Stomach; Gastrointestinal stromal tumor; Carcinoid tumor

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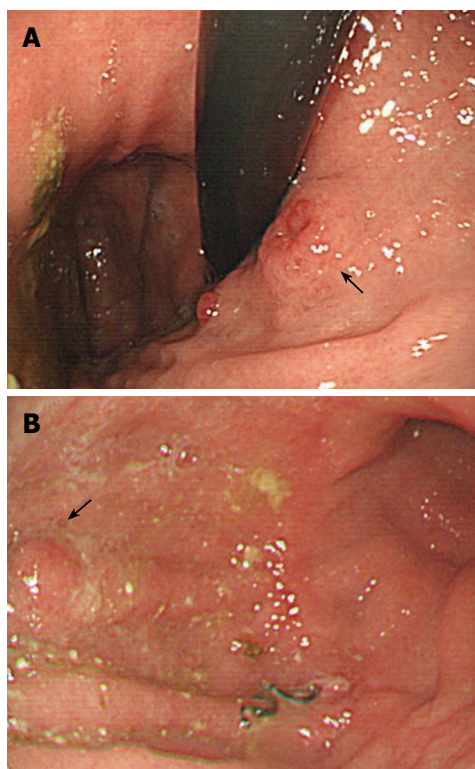


Figure 1 Gastroscopic findings. A: 1.2 cm ulcerated polyp in the cardia (arrow); B: 0.6 cm polyp on the anterior wall of the corpus near the previous anastomosis (arrow).

chromogranin-A, weakly positive for synaptophysin, and negative for CD56, neuron-specific enolase, and cytokeratin 7. Gastric carcinoid was therefore diagnosed. There was inflammation, but no *Helicobacter pylori* (*H. pylori*) was found. The normal gastric body glands were atrophic and replaced by pyloric and intestinal glands. Abdominal ultrasonography, computerized tomography, barium small bowel series, and colonoscopy were performed to exclude other possible gastrointestinal tumors; there were no other abnormalities found. The patient denied any symptoms of diarrhea, palpitation, facial flushing, or weight loss, and there was no family history suggestive of similar disorders or multiple endocrine syndromes. Her abdominal examination was normal except for the previous surgical scar. The fasting serum gastrin level was 1920 pg/mL (normal 25-125 pg/mL). The 24-h urinary 5-HIAA level was normal. These findings were consistent with a type 1 hypergastrinemic gastric carcinoid tumor. Total gastrectomy was performed because the patient was concerned about further recurrences of gastric tumors. The pathologist reported two carcinoid tumors in the cardia and lower corpus near the previous anastomosis. There was no immunohistochemical evidence of GIST in the two carcinoid tumors (Figure 2). One dissected lymph node had metastatic carcinoid tumor. A 0.6 cm focal GIST was also found on the lesser curvature; and there was no carcinoid component on immunohistochemical staining (Figure 3). The pathologic slides from the first surgery were reviewed and immunohistochemical staining

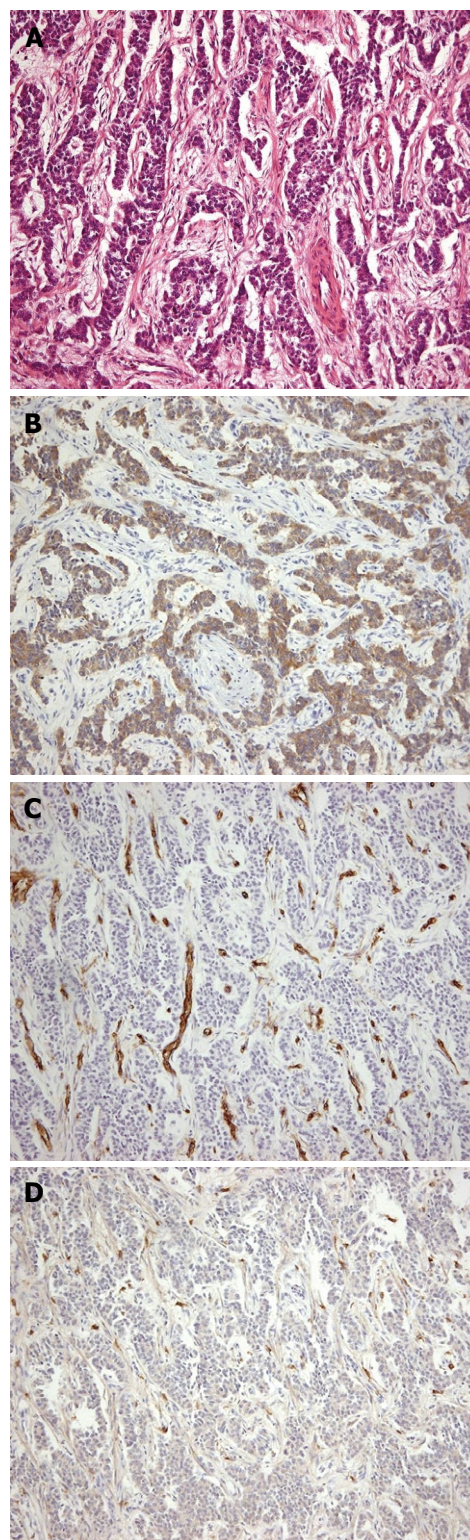


Figure 2 Carcinoid tumor. A: Uniform round-to-oval cells in trabeculae, nests, and gland-like structures (hematoxylin and eosin, × 100); B: Positive immunohistochemical staining for synaptophysin (× 100); C: Negative immunohistochemical staining for CD 34 (× 100); D: Negative immunohistochemical staining for CD 117 (× 100).

repeated, which showed no evidence of carcinoid tumor. Three weeks after the total gastrectomy, the patient's fasting gastrin level had returned to normal (72.40 pg/mL). She recovered uneventfully and has remained well for six months after surgery.

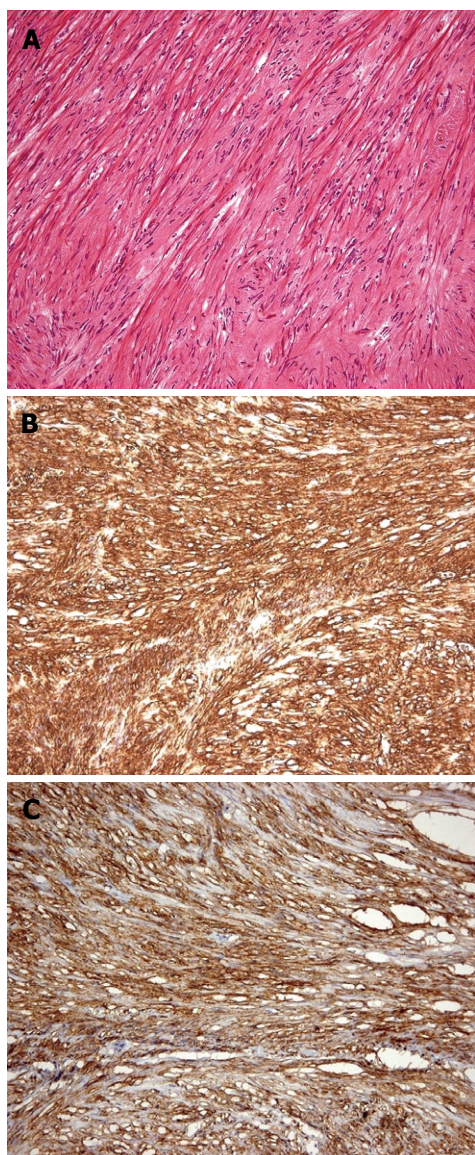


Figure 3 Recurrent gastrointestinal stromal tumor. A: Composed of spindle cells (hematoxylin and eosin $\times 100$); B: Positive immunohistochemical staining for CD34 ($\times 100$); C: Positive immunohistochemical staining for CD 117 ($\times 100$).

DISCUSSION

This patient had metachronous gastric GIST and gastric carcinoid tumors, a previously unreported phenomenon. The tumors were confirmed by pathologic examination, including immunohistochemical studies, to be totally distinct neoplasms.

Gastrointestinal carcinoids comprised 73% of all carcinoid tumors in a large series, the most common sites being in the small bowel, appendix and rectum^[6]. Gastric carcinoids are less common, reported as being only 8.7% of enteric carcinoids. Among all gastric cancers, carcinoids are quite rare, with a reported incidence of only 0.35%-1.77%^[7]. Gastric carcinoid tumors may present with anemia caused by bleeding from an ulcerative mass, abdominal pain, or with a classical carcinoid syndrome. Histologically, the tumors have uniformly round cells growing in rosettes, cords, or trabecular patterns. Immunohistochemical stains are usually positive for chromogranin A or C and

synaptophysin but negative for CD117. The latter marker distinguishes carcinoid from GIST, which is positive for CD117.

Gastric carcinoid tumors can be classified into four types: type 1, with enterochromaffin-like cells, chronic atrophic gastritis, achlorhydria, hypergastrinemia, and often pernicious anemia; type 2, with enterochromaffin-like cells, Zollinger-Ellison syndrome, multiple endocrine neoplasia type 1, and hypergastrinemia; type 3, with enterochromaffin-like cells but which is gastrin-independent and occurs sporadically; and type 4, with miscellaneous non-enterochromaffin-like endocrine cells^[8-10]. The prognosis of types 1 and 2 gastric is better than that of types 3 or 4 gastric carcinoid tumors. The 5-year and 10-year survival rates were 96.1% and 73.9% for type 1 disease, compared with only 33.3% and 22.2% for type 4 gastric carcinoid tumors^[9]. For type 1 gastric carcinoids, limited surgery, such as endoscopic mucosal resection^[11] or partial gastrectomy is adequate, along with appropriate treatment for the hypergastrinemia^[9,10,12]. Radical resection is recommended for types 3 and 4^[9,10]. Our patient had type 1 gastric carcinoid with hypergastrinemia but no associated symptoms, such as Zollinger-Ellison syndrome or multiple endocrine neoplasia type 1. If she had had only the type 1 gastric carcinoid, more conservative surgery might have been considered. The history of the previous GIST, however, influenced the choice of total gastrectomy.

In a retrospective review of 200 patients with gastrointestinal GIST (with 78% or 39% in the stomach), DeMatteo *et al*^[13] reported an overall 5-year survival rate of about 35%, but it depended on the extent of resection. For patients undergoing complete resection, the 5-year survival was 54%, with those patients surviving a median of 66 mo compared with 22 mo for those who had incomplete resection. The recurrence rate was 40% (median 24 mo' follow-up). The median disease-specific survival for patients with local recurrence was 12 mo. Metastases usually occurred with tumors larger than 5 cm or with a mitotic index of more than 10 mitoses per 50 high power fields. The authors recommended complete surgical resection for GIST if possible.

In estimating our patient's prognosis, it is difficult to know if it should be predicted based on the locally recurrent GIST or the carcinoid metastatic to a lymph node. Carcinoid metastases to regional lymph nodes cannot be reliably predicted by tumor size or depth of invasion, their impact on survival is uncertain^[14], and there is no recommended adjuvant treatment. However, type 1 carcinoid is fairly indolent and is less likely than the GIST to limit our patient's survival. We intend to monitor the patient at 6-12 mo intervals with computed tomography for GIST and using plasma chromogranin measurements and octreoscan for carcinoid. Adjuvant therapy with imatinib is an option for treating residual GIST, with evaluation of *c-Kit* and *PDGFR4* mutations a useful tool to predict efficacy^[15,16]. There was also a clinical trial conducted by Yao *et al*^[17], testing the imatinib in 27 carcinoid patients with a modest clinical

response of median overall survival of 36 mo. However, our patient's recurrent GIST was totally removed by gastrectomy, and the mitotic index was low. There would be no way of assessing treatment response and therefore no clear benefit, so imatinib is not indicated in this patient unless the GIST is found to recur on follow-up.

In conclusion, our patient had an apparently unique combination of two unusual gastric tumors. Accurate diagnosis of her GIST and carcinoid tumors, based on immunohistochemical studies, was important both for deciding on treatment and follow-up and in estimating prognosis. However, as this case clearly illustrates, a number of individual patient variables affect those decisions and estimates. Inevitably we must rely on clinical judgment which, while informed by what the evidence says about the diseases in general, must also take into account a patient's particular characteristics, needs, and preferences.

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Events Calendar 2008-2009

FALK SYMPOSIA 2008
 January 24-25, Frankfurt, Germany
 Falk Workshop: Perspectives in Liver Transplantation

International Gastroenterological Congresses 2008
 February 14-16, Paris, France
 EASL-AASLD-APASL-ALEH-IASL Conference Hepatitis B and C virus resistance to antiviral therapies
www.easl.ch/hepatitis-conference

February 14-17, Berlin, Germany
 8th International Conference on New Trends in Immunosuppression and Immunotherapy
www.kenes.com/immuno

February 28, Lyon, France
 3rd Congress of ECCO - the European Crohn's and Colitis Organisation
 Inflammatory Bowel Diseases 2008
www.ecco-ibd.eu

February 29, Québec, Canada
 Canadian Association of Gastroenterology
 E-mail: general@cag-acg.org

March 10-13, Birmingham, UK
 British Society of Gastroenterology Annual Meeting
 E-mail: BSG@mailbox.ulcc.ac.uk

March 14-15, HangZhou, China
 Falk Symposium 163: Chronic Inflammation of Liver and Gut

March 23-26, Seoul, Korea
 Asian Pacific Association for the Study of the Liver
 18th Conference of APASL: New Horizons in Hepatology
www.apaslseoul2008.org

March 29-April 1, Shanghai, China
 Shanghai-Hong Kong International Liver Congress
www.livercongress.org

April 05-09, Monte-Carlo (Grimaldi Forum), Monaco
 OESO 9th World Congress, The Gastro-esophageal Reflux Disease: from Reflux to Mucosal Inflammation-Management of Adeno-carcinomas
 E-mail: robert.giuli@oeso.org

April 9-12, Los Angeles, USA
 SAGES 2008 Annual Meeting - part of Surgical Spring Week
www.sages.org/08program/html/

April 18-22, Buenos Aires, Argentina
 9th World Congress of the International Hepato-Pancreato Biliary Association
 Association for the Study of the Liver
www.ca-ihpba.com.ar

April 23-27, Milan, Italy
 43rd Annual Meeting of the European Association for the Study of the Liver
www.easl.ch

May 2-3, Budapest, Hungary

Falk Symposium 164: Intestinal Disorders

May 18-21, San Diego, California, USA
 Digestive Disease Week 2008

May 21-22, California, USA
 ASGE Annual Postgraduate Course
 Endoscopic Practice 2008: At the Interface of Evidence and Expert Opinion
 E-mail: education@asge.org

June 4-7, Helsinki, Finland
 The 39th Nordic Meeting of Gastroenterology
www.congex.com/ngc2008

June 5-8, Sitges (Barcelona), Spain
 Semana de las Enfermedades Digestivas
 E-mail: sepd@sepd.es

June 6-8, Prague, Czech Republic
 3rd Annual European Meeting: Perspectives in Inflammatory Bowel Diseases
 E-mail: meetings@imedex.com

June 10-13, Istanbul, Turkey
 ESGAR 2008 19th Annual Meeting and Postgraduate Course
 E-mail: fca@netvisao.pt

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 16th International Congress of the European Association for Endoscopic Surgery
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June 13-14, Amsterdam, Netherlands
 Falk Symposium 165: XX International Bile Acid Meeting. Bile Acid Biology and Therapeutic Actions

June 13-14, Prague, Czech Republic
 Central and Eastern European Conference on Colorectal "Cancer" Screening, Prevention and Management
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 10th World Congress on Gastrointestinal Cancer
 Imedex and ESMO
 E-mail: meetings@imedex.com

June 25-28, Lodz, Poland
 Joint Meeting of the European Pancreatic Club (EPC) and the International Association of Pancreatologists (IAP)
 E-mail: office@epc-iap2008.org
www.e-p-c.org
www.pancreatology.org

June 26-28, Bratislava, Slovakia
 5th Central European Gastroenterology Meeting
www.ceurgem2008.cz

July 9-12, Paris, France
 ILTS 14th Annual International Congress
www.iltis.org

September 10-13, Budapest, Hungary
 11th World Congress of the International Society for Diseases of the Esophagus
 E-mail: isde@isde.net

September 13-16, New Delhi, India
 Asia Pacific Digestive Week
 E-mail: apdw@apdw2008.net

III FALK GASTRO-CONFERENCE
 September 17, Mainz, Germany

Falk Workshop: Strategies of Cancer Prevention in Gastroenterology

September 18-19, Mainz, Germany
 Falk Symposium 166:
 GI Endoscopy - Standards & Innovations

September 18-20, Prague, Czech Republic
 Prague Hepatology Meeting 2008
www.czech-hepatology.cz/phm2008

September 20-21, Mainz, Germany
 Falk Symposium 167:
 Liver Under Constant Attack - From Fat to Viruses

September 24-27, Nantes, France
 Third Annual Meeting
 European Society of Coloproctology
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October 18-22, Vienna, Austria
 16th United European Gastroenterology Week
www.negf.org
www.acv.at

October 22-25, Minnesota, USA
 Anstralian Gastroenterology Week 2008
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- 3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

Organization as author

- 4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462]

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- 5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764]

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- 6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303]

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- 9 Outreach: Bringing HIV-positive individuals into care. *HRS/A Careaction* 2002; 1-6 [PMID: 12154804]

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- 10 **Sherlock S**, Dooley J. Diseases of the liver and biliary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

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- 11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

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- 12 **Breedlove GK**, Schorffheide AM. Adolescent pregnancy. 2nd ed. Wiczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

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- 13 **Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

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- 14 **Christensen S**, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

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- 15 Morse SS. Factors in the emergence of infectious diseases. *Emerg Infect Dis* serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: <http://www.cdc.gov/ncidod/EID/eid.htm>

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- 16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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