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*EDITORIAL*

# Growth factor receptors and related signalling pathways as targets for novel treatment strategies of hepatocellular cancer

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## Abstract

Growth factors and their corresponding receptors are commonly overexpressed and/or dysregulated in many cancers including hepatocellular cancer (HCC). Clinical trials indicate that growth factor receptors and their related signalling pathways play important roles in HCC cancer etiology and progression, thus providing rational targets for innovative cancer therapies. A number of strategies including monoclonal antibodies, tyrosine kinase inhibitors ("small molecule inhibitors") and antisense oligonucleotides have already been evaluated for their potency to inhibit the activity and downstream signalling cascades of these receptors in HCC. First clinical trials have also shown that multi-kinase inhibition is an effective novel treatment strategy in HCC. In this respect sorafenib, an inhibitor of Raf-, VEGF- and PDGF-signalling, is the first multi-kinase inhibitor that has been approved by the FDA for the treatment of advanced HCC. Moreover, the serine-threonine kinase of mammalian target of rapamycin (mTOR) upon which the signalling of several growth factor receptors converge plays a central role in cancer cell proliferation. mTOR inhibition of HCC is currently also being studied in preclinical trials. As HCCs represent hypervascularized neoplasms, inhibition of tumour vessel formation *via* interfering with the VEGF/VEGFR system is another promising approach in HCC treatment. This review will summarize the current status of the various growth factor receptor-based treatment strategies and in view of the multitude of novel targeted approaches, the rationale for combination therapies for advanced HCC treatment will also be taken into account.

## INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common malignancy worldwide. With an alerting 5 years relative survival rate of about 7% HCC is estimated to cause more than half a million deaths annually. HCC is the third most common cause of cancer deaths. The disease is most prevalent in Eastern and Southeastern Asia, and Middle Africa, with more than half of the patients being reported from China[1]. However, also in the developed countries the incidence of HCC dramatically increased in the past decades, mainly due to the increasing prevalence of chronic hepatitis C[2].

HCC is one of the few cancers with well-defined major risk factors. In Western countries > 80% of HCC develop in livers with cirrhosis mainly due to chronic hepatitis C, alcohol abuse, chronic hepatitis B or hemochromatosis. Especially in developed countries there is increasing concern regarding the epidemic of obesity which is associated with type 2 diabetes and other features of the metabolic syndrome and which frequently leads to non-alcoholic steatohepatitis (NASH). Here NASH may become to be one of the major causes of cirrhosis; diabetes and NASH are risk factors for developing HCC[2-4]. Rarer causes are cirrhosis due to hemochromatosis, autoimmune liver diseases or congenital disorders of metabolism. Cirrhosis in a setting of chronic liver cell injury, with inflammation, hepatocyte necrosis and regeneration, is a particular breeding ground for hepatocyte dedifferentiation and HCC[5]. In developing

countries, HCC frequently arises in non-cirrhotic livers, mostly on the basis of congenital infection with the hepatitis B virus which acts as mutagen due to insertion in the human genome and/or on the basis of aflatoxin exposure from contaminated food[2,4].

Unfortunately, the majority of patients suffer from advanced HCC at presentation. Therefore, curative treatment like local ablation, surgical resection or liver transplantation can be achieved in only a minority of HCC patients[6]. Local tumour destruction, chemoembolisation or systemic therapy are the treatment options of advanced HCC. Apart from transarterial chemoembolisation, which improves survival in well-selected patients with unresectable HCC, conventional palliative treatment options do not appear to improve overall outcome[5,6]. A recent meta-analysis of Simonetti and coworkers, who evaluated the results of randomized clinical trials of systemic and regional chemotherapy of HCC patients confirmed the disappointing results and revealed that nonsurgical therapies are more or less ineffective and do not prolong the survival of HCC patients, while further compromising quality of life[7].

Effective palliative treatment is hampered by the fact that advanced HCC represents a tumour entity which is extremely resistant to radiotherapy and conventional chemotherapy[8]. Moreover, the existing conventional chemotherapeutics are more or less non-selective cytotoxic drugs with significant systemic side-effects. Importantly, as most patients with advanced HCC have compromised liver function aggressive medical therapy regimens can not be applied. Thus, usually no effective therapy can be offered to these patients.

Because of the lack of any survival benefit of treatment with conventional drugs, new agents and novel therapeutic strategies are urgently needed to improve palliative treatment, prolong life expectancy and improve quality of life in patients with advanced HCC.

## POTENTIAL TARGETS FOR FUTURE HCC THERAPIES

Growth factors and their related receptors are interesting targets for future therapeutic approaches. During foetal life, a large number of growth factors including the epidermal growth factor (EGF), insulin-like growth factors (IGFs), the hepatocyte growth factor (HGF), the vascular endothelial growth factor (VEGF), the fibroblast growth factor (FGF), the platelet-derived growth factor (PDGF) and the transforming growth factors -α and -β (TGF-α, TGF-β) are produced in the liver. In the adult normal liver many of them decline or are even absent. On the other hand adult hepatocytes are able to upregulate the production of particular growth factors like EGF, TGF-α, IGFs and VEGF, when liver regeneration is required after injury or damage[9,10]. This normally transient upregulation is dysregulated in the chronic injured liver leading to sustained mito-oncogenic signalling. Thus, dysregulation of the growth factor production and growth factor receptor signalling of adult hepatocytes plays an important role in hepatocarcinogenesis.

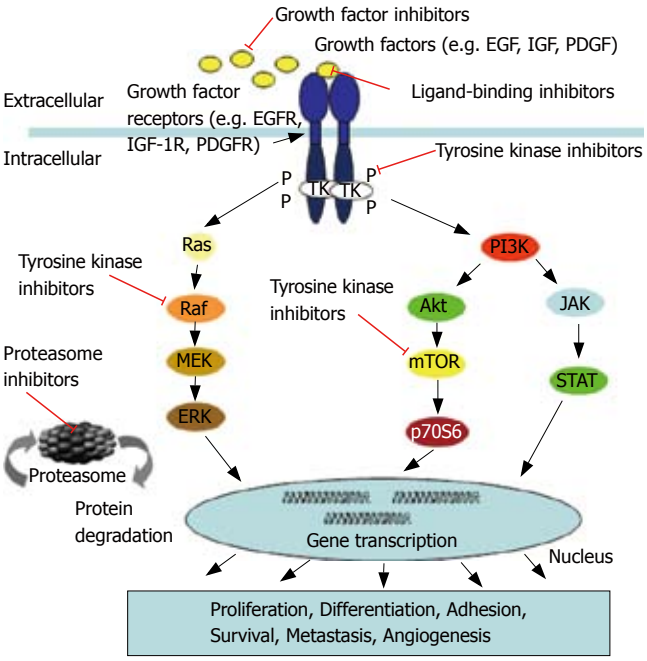


**Figure 1** Major growth factor receptor signalling pathways in HCC.

Furthermore, members of the fibroblast growth factor and platelet-derived growth factor families, FGFs and PDGF play important roles in promoting liver fibrosis and HCC growth[11,12]. Like HGF, these growth factors are produced and released from non-hepatocyte sources like activated hepatic stellate cells, myofibroblasts, endothelial cells, Kupffer cells and bile duct epithelia and do also contribute to hepatocarcinogenesis.

## GROWTH FACTOR RECEPTOR RELATED SIGNALLING PATHWAYS IN HCC CELLS

In the last decade some of the relevant pathways in cancer biology have been deciphered and there is emerging evidence that particularly growth factor receptors and their related downstream signalling pathways play a pivotal role in the development and maintenance of various cancers including HCC. Among the most critical cellular signalling pathways which support hepatocarcinogenesis are the receptor tyrosine kinase-activated pathways which include the rat sarcoma/rat sarcoma-activated factor (raf) /mitogen activated protein kinase/extracellular regulated kinase kinase/extracellular regulated kinase pathway (Ras/Raf/MEK/ERK), the Janus kinase/signal tranducers and activator of transcription pathway (JAK/STAT)s, and the phosphatidyl inositide 3 kinase/protein kinase B (AKT) /mammilian target of rapamycin pathway (PI3K/AKT/mTOR) (Figure 1).

### *Ras/Raf/MEK/ERK pathway*

The Ras/Raf/MEK/ERK pathway appears to be one of the most significant cellular signalling sequences in the development and maintenance of hepatocellular cancer. This pathway transduces extracellular signals from ligand-bound tyrosine kinase receptors, such as the epidermal growth factor receptor (EGFR), the insulin-like growth

factor receptor (IGFR), the vascular endothelial growth factor receptor (VEGFR) or the platelet-derived growth factor receptor (PDGFR) to the nucleus in a series of specific phosphorylation events, starting with the activation of Ras which in turn activates serine threonine kinases of the Raf-family[1]. Activated Raf phosphorylates MEK 1/2 kinases which finally activate the extracellular regulated kinases ERK 1/2. Once activated, ERK 1/2 translocates to the nucleus where it acts as a regulator of gene expression of various proteins, including those for cell cycle progression, apoptosis resistance, extracellular matrix (ECM) remodeling, cellular motility angiogenesis and drug resistance[13]. Dysregulation of this crucial pathway occurs due to oncogenic transformation of Ras and Raf isoforms, or overexpression and/or overactivation of the Ras and Raf genes. In a recent study overexpression of the *Raf*-1 gene was shown in 50% of HCC biopsies, while increased activation of the Raf-1 protein was found even in 100% of the $n = 30$ evaluated HCC biopsies[14]. However, significant contribution of the proliferative Ras pathway to the development of HCC has long been a matter of debate. Previous studies suggested that activation of the Ras pathway might only be important in rodent, but not in human HCC, because of the low incidence of Ras gene mutations found in human HCC, while activation of the Ras cascade also occurred in the presence of wild-type Ras[15]. Recently, the components of the Ras cascade in human HCCs were characterized, demonstrating a downregulation or loss in the expression of specific members of the RAS inhibitor family. Among them the RAS association family 1 gene A (RASSF1A) and its homologue NORE1A in 100% of the $n = 35$ examined HCC[16]. The inactivation of these inhibitors resulted in a persistent activation of the Ras pathway and the authors suggested that the use of Ras inhibitors may thus be an interesting therapeutic modality for future treatment of HCC.

### JAK/STAT pathway

The same holds true for the JAK/STAT pathway which plays an important role in cellular processes like differentiation, proliferation, and apoptosis[17]. STATs are latent in the cytoplasm and become activated through tyrosine phosphorylation which typically occurs through JAKs or growth factor receptor tyrosine kinases. Activated STATs enter the nucleus and serve as transcription factors. As to apoptosis and cell cycle related genes the transcriptional changes induced by STATs are similar to those described for ERK1/2.

In normal cells, ligand-dependent activation of STATs is transient, but in tumours the STAT proteins (in particular STAT-1, -3 and -5) are often constitutively activated[18,19]. This constitutive activation is partly due to inactivation of specific STAT inhibitors, the suppressors of cytokine signalling (SOCS), which normally balance and terminate STAT activity[20,21]. Thus, loss of activation of the STAT inhibitors such as cytokine-inducible SH2-protein (CIS), SOCS1, SOCS2, SOCS3, and SH2-containing phosphatases (SHP1) was shown to account for the constitutive activation of antiapoptotic and mitogenic STAT-3 and -5 in HCC[16].

In terms of both the Ras and the JAK/STAT pathway it may not be the increase in gene mutations of the respective pathway proteins, but the state of activation of these pathways due to an imbalanced interplay of activators and inhibitors, which accounts for the pivotal role of these pathways in HCC.

### PI3K/AKT/mTOR pathway

The activated PI3K/AKT/mTOR pathway has only recently emerged as a novel contributor to (HCC) tumour development. PI3K associates with the intracellular domain of several growth factor receptors. Upon activation PI3K triggers the generation of phosphatidylinositol 3,4,5-triphosphate ($PIP_3$) which provokes the subsequent activation of AKT, a serine/threonine kinase which regulates multiple cellular target proteins. Among these proteins is proapoptotic BAD, which becomes inactivated by phosphorylation, and also the mammalian target of rapamycin (mTOR) subfamily of proteins, which become activated by AKT. mTOR proteins regulate the phosphorylation of p70 S6 serine-threonine kinase and the translational repressor protein PHAS-1/4E-BP. Both proteins regulate the translation of proliferation- and angiogenesis-relevant proteins, such as c-myc, cyclin-D1, ornithine decarboxylase, hypoxia-induced factor 1-$\alpha$, and are indirectly involved in the expression of VEGF[1,22].

In nontransformed cells the PI3K/AKT/mTOR pathway is controlled by the phosphatase and tensin homolog deleted on chromosome ten (PTEN), a tumour suppressor which inhibits this pathway by reversing the PI3K reaction and blocking AKT activation. Mutation or silencing of the PTEN gene leads to activation of the pathway and promotes carcinogenesis. PTEN expression is reduced or absent in almost half of the studied HCCs, and hepatocyte-specific abrogation of PTEN expression in mice results in the development of HCC[23]. Thus, constitutive activation of this pathway can be due to enhanced stimulation of growth factor receptors, like EGFR and IGFRs, but can also result from decreased PTEN expression[1]. In non-HCC tumour models loss of PTEN expression has been demonstrated to negatively influence the sensitivity towards EGFR-TK inhibition by gefitinib[24]. Thus, it will be interesting to evaluate if this also holds true for HCC. If so, PTEN expression can serve as a novel marker for predicting the response to tyrosine kinase inhibition-based treatment strategies in HCC and therapeutic strategies in which normal PTEN expression can be restored, should be an attractive approach for combined therapeutic strategies in HCC treatment in the future.

Moreover, recent work by Boyault and coworkers demonstrated that in specific subgroups of HCC patients, namely those that are infected with low copy number hepatitis B virus (HBV) and concomitant overexpression of genes expressed in fetal liver, as well as those infected with a high copy number of HBV, but concomitant mutations in the catalytic domain of the phosphoinositide-3 kinase (*PIK3CA*) and the tumor suppressor protein 53 (*TP53*), there is a frequent upregulation of AKT expression and activation, rendering

**Table 1 Agents for anti-EGFR-based therapy of solid tumours**

| Name | Target | Mechanism | Current status |
|---|---|---|---|
| Small molecule inhibitors | | | |
| Gefitinib (Iressa) | EGFR | Reversibly acting tyrosine kinase inhibitor | Approved for NSCLC with restricted indications<br>Phase I for HCC[133] |
| Erlotinib (Tarceva) | EGFR | Reversibly acting tyrosine kinase inhibitor | Approved for NSCLC and pancreatic cancer<br>Phase Ⅱ for hepatocellular cancer[33] |
| EKB-569 | EGFR | Irreversibly acting tyrosine kinase inhibitor | Phase Ⅰ/Ⅱ for colorectal cancer[134]<br>Phase Ⅱ for NSCLC[135] |
| Lapatinib (Tykerb) | EGFR, erbB2 | Reversibly acting tyrosine kinase inhibitor | Phase Ⅲ for breast cancer<br>Phase Ⅱ for HCC[136] |
| Canertinib (CI-1033) | Pan-erbB | Irreversibly acting tyrosine kinase inhibitor | Phase Ⅱ for SCC and ovarian cancer[137] |
| BMS-599626 | EGFR, erbB2 | Reversibly acting tyrosine kinase inhibitor | Phase Ⅱ for HCC [138] |
| Monoclonal antibodies | | | |
| Cetuximab | EGFR | | Approved for colorectal cancer<br>Phase Ⅲ for head and neck cancer, NSCLC and pancreatic cancer<br>Phase Ⅱ for HCC[139] |
| Trastuzumab | erbB2 | | Approved for breast cancer |
| ABX-EGF | EGFR | | Phase Ⅲ for colorectal-, head and neck-, and renal cell cancer |
| Matuzumab (EMD 72000) | EGFR | | Phase I/Ⅱ for NSCLC[140], ovarian-[141], pancreatic cancer[142] |

these patients especially susceptible to therapeutic approaches that inhibit the AKT-pathway[25].

Due to the importance of the above-described signalling pathways linked to growth factor receptors in the development and maintenance of (hepatocellular) cancer, several attempts have been undertaken to develop specific inhibitors which either block the communication of growth factors and their cognate receptors using antibodies or growth factor trapping decoy receptors, or by interrupting the transmission of growth factor receptor which signals to the respective downstream signalling cascades by membrane permeable small molecule inhibitors which block the intrinsic receptor tyrosine kinase activity. The following section will provide a concise overview of selected agents which are currently in the development and testing for the targeted treatment of solid tumours and HCC.

### *EGFR-based strategies*

The crucial role of epidermal growth factor receptor (EGFR) in tumour proliferation and its overexpression in several solid tumours have provided the rationale for targeting and interrupting this key signalling network. EGFR blockade through monoclonal antibodies and tyrosine kinase inhibitors has translated into promising evidence of clinical benefit in gastrointestinal tumours, particularly colorectal cancer[26]. EGFR is expressed in a high proportion of HCCs, and EGFR-inhibitors, such as the monoclonal antibody cetuximab or the tyrosine kinase inhibitors gefitinib, erlotinib or ANAPD have been shown to inhibit HCC growth and metastasis formation *in vitro*[27-31] and *in vivo*[32]. Recently, Philips and coworkers conducted a phase Ⅱ trial with erlotinib for advanced HCC and could demonstrate very encouraging results, as they observed good response rates in approx. One third of the treated patients and a prolonged survival with mild and tolerable side effects after treatment with a dose of 150 mg/d[33].

Despite the encouraging findings on the general suitability of anti-EGFR-based-approaches for the treatment of HCC, only few clinical trials have been conducted so far. Most of our current knowledge on the clinical benefit of anti-EGFR-based therapies originates from studies on other tumour entities, such as colorectal cancer, renal cell carcinoma and non-small cell lung cancer (NSCLC). Nevertheless, at present several clinical trials evaluate the efficacy of anti-EGFR-interventions for the treatment for HCC (www.clinical-trials.gov), and based on the data that are available so far there is hope that anti-EGFR mono- or combination therapies will qualify for improving the treatment of advanced HCC in the near future. Currently, another phase Ⅱ trial is conducted in patients with advanced HCC which evaluates the efficacy of a combination of erlotinib and the anti-angiogenic VEGF-blocking antibody bevacizumab (NIH, NCT00365391). Dual-targeting of the HCC cells and their nutrient supply *via* the surrounding vasculature may improve the antitumoural effects as compared to monotherapy with either erlotinib or bevacizumab alone[34].

Thus the majority of the currently tested anti-EGFR-based approaches are increasingly combined either with conventional cytostatics or with other targeted-agents[28,29,35,36]. The rationale for applying combination therapies is the existence of multilevel receptor cross-stimulation or of redundant signalling pathways that lead to neoplasia. Blocking only one of these pathways allows others to act as salvage or escape mechanisms for cancer cells. Preclinical evidence of synergistic antitumour activity achievable by combining targeted agents that block multiple signalling pathways has recently emerged[37-41]. The multi-target approach can be accomplished by using either combinations of selective agents or agents which interfere with various targets[42]. Table 1 shows the current status of anti-EGFR-strategies for the treatment of solid tumours including HCC.

### *IGF/IGFR-based strategies*

There is compelling evidence that both insulin-like growth factors IGF-Ⅰ and -Ⅱ and their receptor tyrosine kinase, IGF-1R, are involved in the development and

**Table 2 Agents for anti-IGF-1R-based cancer treatment**

| Name | Target | Mechanism | Current status |
|---|---|---|---|
| Small moelcule inhibitors | | | |
| INSM-18 | IGF-1R and HER2 | Substrate competitive inhibitor | Phase Ⅰ[44] |
| NVP-AEW541 | IGF-1R | ATP-competitive inhibitor | Preclinical[63] |
| NVP-ADW742 | IGF-1R | ATP-competitive inhibitor, activation of proapoptotic pathways | Preclinical[62] |
| BMS-536924 | IGF1R and IR | ATP competitive inhibitor, | Preclinical[143] |
| Cyclolignans | IGFR-1R | IGF competitive inhibitor | Preclinical[144] |
| Antibodies | | | |
| CP-751, 871 | IGF-1R | IGF1R downregulation | Phase I for multiple myeloma<br>Phase Ⅱ for Breast-[145], lung-[146], and prostate[147] cancer |
| A12 | IGF-1R | IGF1R down-regulation, apoptosis, cell cycle arrest | Phase Ⅰ[148] |
| scFv-Fc | IGF-1R | IGF1R downregulation | Preclinical[149] |
| AVE-1642 | IGF-1R | IGF1R downregulation, cell-cycle arrest, induction of apoptosis | Preclinical[69] |

progression of cancer[43-46]. Interaction of IGF-Ⅰ and -Ⅱ with the IGF-1R plays a pivotal role in tumourigenesis, proliferation and spread of many cancers, by promoting cell cycle progression, preventing apoptosis, and by regulating and maintaining the tumourigenic phenotype. A wide variety of tumours including HCC show abnormal, or enhanced expression of IGFs and IGF-1R, which has been correlated with disease stage, reduced survival, development of metastases and tumour de-differentiation[47-49]. In men, obesity and diabetes are clearly associated with an increased risk of HCC, and this seems to be due to alterations in the metabolism of endogenous hormones, including sex steroids, insulin and the IGF/IGFR system. Thus, a promising approach of innovative HCC treatment may be the blockade of the IGF/IGFR, but also the mTOR-signalling system, which is functionally upregulated in HCC cells *in vitro*[50-52] and *in vivo*[47], and which has been shown to exert strong stimulatory effects on the growth of hepatoma cells[48]. In addition to the increased expression of IGF-1R and IGFs, a simultaneous reduction of IGF binding protein expression (IGFBP) and enhanced proteolytic cleavage of IGFBPs often occurs. Both mechanisms lead to an excessive increase in the amount of bioactive IGF[50,53] which further enhances the mito-oncogenic effects of IGFR-signalling in HCC and other cancer cells. The expression of IGF-1R is very low in normal hepatocytes that are poorly responsive to IGFs, whereas significant expression is found in Kupffer, endothelial and hepatic stellate cells[50].

Several approaches have demonstrated the therapeutic potential of interfering with IGF-1R mediated signalling *in vitro* and *in vivo*, including the use of IGF-1R blocking antibodies, IGF-1R antisense oligonucleotides or IGF-1R siRNA[54-57].

Recently, we and others introduced the potent and selective IGF-1R tyrosine kinase inhibitor, NVP-AEW541, as promising novel agent for the therapy of several cancers[58-60], including HCC[61]. The antineoplastic properties of NVP-AEW541 and related compounds such as NVP-ADW742[62] have been demonstrated in preclinical studies on Ewing´s sarcoma-bearing mice[63], fibrosarcoma, breast cancer and musculoskeletal carcinoma[64-66]. Specific IGFR-antibodies have also shown to suppress prostate and breast cancer cell growth in a recent preclinical study[67]. The clinically most advanced anti-IGFR antibody is CP-751,871 which is currently being tested in three phase Ⅱ trials for advanced breast cancer, NSCLC and prostate cancer (www.clinical-trials.gov). Importantly, IGFR-inhibition appears to be well-tolerated in the preliminary clinical studies conducted so far[63,68,69]. Safety is important, since IGFR-based inhibition has long been regarded as a high risk intervention, because of the high homology of the IGF-1R receptor with the related insulin-receptor, and the fear that especially IGF-1R-TK inhibitors might do also block the insulin receptor which could lead to insulin resistance and overt diabetes[70]. However, the current *in vivo* studies did not confirm this apprehension, resulting in growing interest in anti-IGFR-based therapies[71].

It is widely accepted that a therapy which inhibits IGF signalling may have to be combined with other therapies to enhance the antiproliferative overall-effect, since crosstalk between the signalling of IGF and other growth factor receptors have already been shown to be able to attenuate the antineoplastic effects of a respective monotherapeutic approach[72]. Thus, we and others could show that IGFR and concomitant EGFR-inhibition or conventional chemotherapy enhances the antineoplastic effect of the respective monotherapies[28,29,61]. Especially, dual-targeting EGFR and IGF-1R is a promising approach for future treatment of HCC. The rationale for this particular combination is derived from observations that in HCC cells the EGFR-system is activated by the IGF/IGFR-system *via* receptor cross-talk leading to mito-oncogenic EGFR-tyrosine kinase activity[73,74]. Thus inhibition of IGF-2-related signalling leads to sensitization of HCC cells to anti-EGFR-treatment with gefitinib[72], and it was postulated that inhibition of IGF/IGF-1R-signalling may not only enhance the effects of gefitinib treatment, but may also help to overcome resistance to anti-EGFR-based therapy of HCC[75]. Table 2 summarizes the most promising IGF/IGFR-targeted agents which are currently under intense investigation in preclinical and early clinical trials.

### *VEGF/VEGFR-based strategies*

VEGF is the key angiogenic factor in tumours. The VEGF gene and protein have been reported to be transcribed, expressed, and secreted by HCC cells[76]. Endothelial

**Table 3 Agents for anti-VEGF/VEGFR-based therapy of solid tumours**

| Name | Target | Mechanism | Current status |
|---|---|---|---|
| Small molecule inhibitors | | | |
| Sunitinib (Sutent) | PDGFR, VEGFR, c-KIT, FLT-3 | Tyrosine kinase inhibitor | Approved for advanced renal cell carcinoma and GIST (with restricted indications)[87,150]<br>Phase I for HCC[151] |
| Zactima (ZD6474) | VEGFR, EGFR | Tyrosine kinase inhibitor | Phase Ⅲ for NSCLC[156]<br>Phase Ⅱ for thyroid cancer[157] |
| Vatalanib (PTK787/ZK 222584) | VEGFR, PDGFR, C-KIT | Tyrosine kinase inhibitor | Phase Ⅱ/Ⅲ for colorectal cancer[152]<br>Phase I for HCC[153] |
| Anti-ligand targeting | | | |
| Bevacizumab | VEGF | VEGF-neutralizing antibody | Approved for colorectal cancer<br>Phase Ⅲ for NSCLC[166]<br>Phase Ⅱ for HCC[154] |
| VEGF trap | VEGF | Soluble decoy receptor which neutralizes all VEGF A isoforms | Phase I for advanced solid tumours[155] |

cells which line tumour vessels express VEGFR-1 and VEGFR-2 which communicate to stimulate each other in a feedback-loop[77]. Given that VEGF protein expression is related to HCC grade[78] and given that the degree of microvascular density correlates with HCC grade[79,80], it is comprehensible that inhibitors of VEGF signalling are promising therapeutic agents for HCC treatment.

Bevacizumab is a humanized murine monoclonal anti-VEGF antibody which has entered the clinic for treatment of cancer. Standard cytostatic treatment plus bevacizumab significantly increased survival in metastatic colorectal cancer compared to standard treatment alone in a phase Ⅲ clinical trial[81] which led to its approval for treatment of colorectal cancer 2005. Comparable results were obtained in a recent phase Ⅲ clinical with bevacizumab for treatment of NSCLC. This study had to be interrupted because of the obvious survival advantage of patients in the bevacizumab arm[82]. Bevacizumab monotherapy is currently being tested in patients with unresectable HCC[83] (National Cancer Institute: NCT00162669). Moreover, a phase Ⅱ trial is currently being conducted testing the efficacy of bevacuzimab in combination with capcetabine and oxaliplatin in patients with advanced HCC. An intermediate evaluation of this trial is encouraging and shows that this combination is tolerable to patients with advanced HCC and cirrhosis[84]. As described earlier, bevacizumab is also currently tested in combination with the EGFR-tyrosine kinase inhibitor erlotinib (see above).

In addition, several agents that inhibit the tyrosine kinase activity of VEGFR have been synthesized by combinatorial chemistry. Recent clinical studies revealed the suppression of HCC growth by vatalanib (PTK787/ZK 222584), which inhibits the activities of VEGFR-1 and -2 and has shown antineoplastic effects in other solid tumours[85,86]. Another interesting agent is the tyrosine kinase inhibitor sunitinib, which inhibits the VEGFR- as well as the PDGF-βR, c-KIT and FLT-3 tyrosine kinases. Sunitinib has been approved for the treatment of renal cell carcinoma[87]. With restricted indications sunitinib is also approved for the therapy of gastrointestinal stromal tumours (GIST)[88] and is currently tested in phase I and Ⅱ trials for HCC (NIH: NCT00361309; NCT00247676).

Another promising approach is the use of dual-targeting tyrosine kinase inhibitors, which inhibit less related tyrosine kinases, such as NVP-AEE788 or zactima (ZD6474) which target both the VEGFRs and the EGFR. In recent *in vivo* studies of non-HCC tumour models (colon, cholangiocarcinoma, prostate, NSCLC) NVP-AEE788 displayed significant antineoplastic efficacy. These agents can inhibit both tumour cell proliferation and survival by blocking hepatoma EGFR and angiogenesis by inhibiting endothelial VEGFR. These promising recent results warrant further evaluation in clinical trials[89-92]. For zactima successful testing in clinical trials has already been reported for non-HCC tumour entities like NSCLC and thyroid cancer[93,94]. Table 3 summarizes the current status of anti-VEGF/VEGFR-based approaches in the treatment of solid tumours including HCC.

## OTHER MULTI-KINASE AND GROWTH FACTOR RECEPTOR INDEPENDENT INHIBITORS

### *Multi-kinase inhibition*

The novel bi-aryl urea sorafenib is an orally available multi-kinase inhibitor which targets kinases of wild-type B-Raf, mutantV559EB-Raf and C-Raf, thus blocking tumour growth. Furthermore, sorafenib potently inhibits receptor tyrosine kinases involved in angiogenesis, including human vascular endothelial growth factor receptors-2 and -3 (VEGFR-2/-3) and PDGF-βR. The principal mechanism of action of sorafenib is the competitive inhibition of ATP-binding to the catalytic domains of the respective kinases[95]. However, the fact that sorafenib is an oral multi-kinase inhibitor, with effects on several molecular targets in addition to the Raf isoforms makes it difficult to determine which of these targets contributes most to its anti-tumour activity in particular tumour types.

A recent phase Ⅱ HCC clinical trial, which identified an association between high baseline tumour p-ERK levels and improved response to sorafenib, suggests that inhibition of the Raf/MEK/ERK pathway is central to sorafenib's mode of anti-tumour action in HCC[96]. If this generally holds true for HCC remains to be determined. In other tumour entities the antineoplastic potency of

sorafenib appears to be mainly due to its antiangiogenic activity[97,98].

It is of particular clinical importance to have reliable markers to individually predict the treatment outcome. It has been suggested that rash, which is commonly associated with EGF-pathway inhibition, could be predictive of treatment outcome, and that the onset of rash could be used for optimal dose titration[99]. This might also be effective in treatment with sorafenib, as it is an inhibitor of Raf kinase, which is a downstream effector molecule of the EGFR signalling pathway. A recent report combining data from four phase I trials supported this hypothesis. Patients receiving sorafenib dosed at or close to the recommended dose of 400 mg bid, and experiencing skin toxicity and/or diarrhea, had a significantly increased time to progression compared with patients without such toxicity[100].

Sorafenib inhibits the proliferation of a variety of human cancer cell lines and retards tumour growth in related xenograft models of NSCLC, breast, colon and pancreas carcinoma[13,101,102]. Sorafenib is also active in otherwise fairly therapy resistant cholangiocarcinoma cells. Here, it over-additively enhanced the antineoplastic effects of cytostatics such as doxorubicin or the histone deacetylase inhibitor MS-275 and acts synergistically with IGFR blockade[103,104]. Recent *in vitro* studies by our group confirmed the synergistic antiproliferative effects of a combination treatment with sorafenib and MS-275 in hepatocellular carcinoma models. Proliferation studies, with either Hep-G2 or Huh-7 cells, resulted in half-maximal growth inhibition at a sorafenib concentration of 1.6 ± 0.3 μmol/L (Hep-G2) and 4.4 ± 0.2 μmol/L (Huh-7), respectively. The $IC_{50}$ of MS-275 amounted to 1.2 ± 0.1 μmol/L in Hep-G2 cells and 0.9 ± 0.2 μmol/L in Huh-7 cells. Co-application of sub-$IC_{50}$ concentrations of sorafenib and MS-275 for three days resulted in significant over-additive growth inhibition of Huh-7 cells, while in Hep-G2 cells a rather additive growth inhibitory effect was observed (Figure 2). Our data support the idea of dual-targeting hepatocellular carcinoma cells for enhanced treatment efficacy and show that multi-kinase inhibition plus histone deacetylase inhibition appear to be a promising combination, warranting further elucidation in clinical trials.

A series of clinical studies have tested sorafenib's antineoplastic potency in cancer patients. Phase I trials showed a favourable safety profile of 400 mg sorafenib administered twice daily for 12 wk in patients with advanced solid tumours (e.g. colon, ovary, breast, pancreas, kidney)[100,105]. Promising antitumour activities of sorafenib were observed in a phase Ⅱ clinical study of patients with advanced melanoma[106]. Most encouraging results were seen in phase Ⅱ and Ⅲ trails of patients with metastatic renal cell carcinoma (RCC) which led to approval in the US for advanced RCC[107].

Sorafenib has also been tested for the treatment of advanced HCC in phase Ⅱ and Ⅲ trials. In a phase Ⅱ trial on 137 patients with inoperable HCC the continuous oral application of sorafenib 400 mg bid in 4-wk cycles revealed a significant attenuation of HCC growth in 1/3 of the patients[108] resulting in a further evaluation

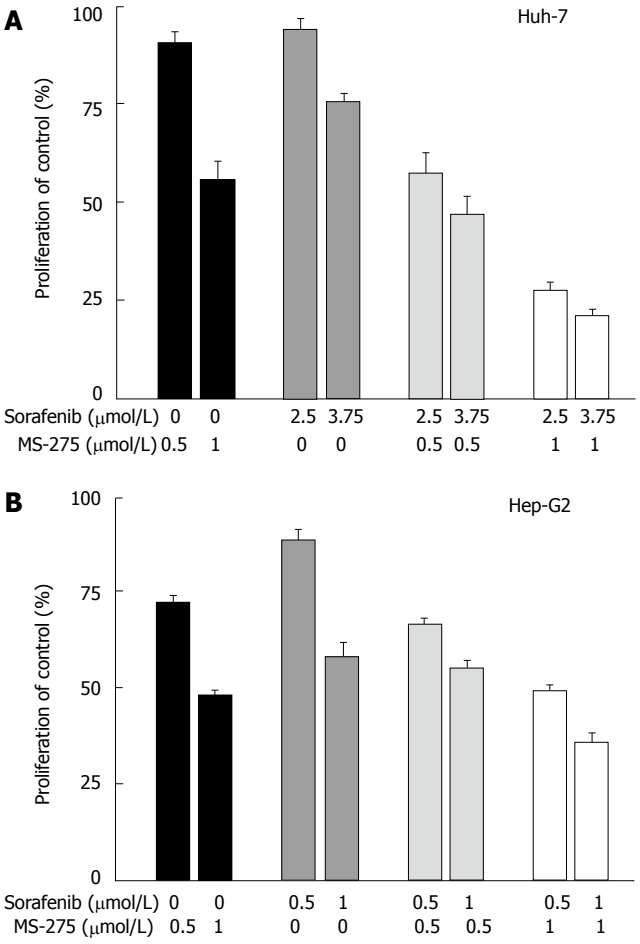


**Figure 2** Antiproliferative effects of sorafenib-based combination treatment. **A**: Huh-7 and **B**: HEP-G2 cells were treated for 72 h with sub-$IC_{50}$ concentrations of sorafenib and the histone deactylase inhibitor MS-275. Combination of both agents resulted in synergistic growth inhibition of Huh-7 cells, while rather additive growth inhibitory effects were observed in HepP-G2 cells (mean ± SEM).

in a randomized double-blinded phase Ⅲ trial with 602 patients with advanced HCC. An interim evaluation of this international multi-center SHARP-study (Sorafenib HCC Assessment Randomized Protocol) led to discontinuation, as the HCC patients treated with sorafenib achieved a significant survival benefit over the placebo-treated controls. Llovet *et al* presented the respective data for the Sharp investigators study group at the ASCO meeting in 2007 and showed that the treatment of advanced HCC patients with sorafenib leads to a 44% improvement in the overall survival as compared to the control group. The median overall survival in the sorafenib treated arm was 10.7 mo *vs* 7.9 mo in the control arm. Moreover, the median time to progression was almost doubled (5.5 mo in the sorafenib arm *vs* 2.8 mo in the control arm). The authors concluded that the effects of sorafenib treatment are clinically meaningful and establish sorafenib as first-line treatment for patients with advanced HCC[109]. Based on these findings, sorafenib has recently gained accelerated approval by the FDA for the treatment of advanced unresectable HCC.

### *mTOR inhibition*

The natural antibiotic rapamycin is a potent inhibitor of mTOR[110]. Recently, three analogues of rapamycin with

superior pharmacokinetic and biological properties have been synthesized and tested in clinical trials for different malignancies. The cell cycle inhibitor-779 (CCI-779, temsirolimus) is a soluble ester analogue. RAD001 (40-O-[2-hydroxyethyl]-rapamycin, everolimus) is an orally bioavailable derivative of rapamycin, and finally AP23573, which is a non-pro-drug analogue of rapamycin. These agents have been tested successfully in early clinical trials for their antineoplastic potency and/or tolerability in various malignancies, such as renal, breast and lung cancers (CCI-779), or are currently being studied in open clinical trials for the treatment of colorectal, endometrial cancer, recurrent or refractory solid tumours, and brain tumours (RAD001, everolimus)[111-113]. AP23573 has been successfully tested in a phase Ⅱ trial in sarcomas[114] and two phase I studies in patients with refractory or advanced solid tumours, showing partial responses and disease stabilisation in individual patients[115].

*In vitro* as well as preclinical *in vivo* data of HCC show, that mTOR inhibition by rapamycin and analogues significantly reduces HCC growth and improves survival primarily *via* antiangiogenic effects[116]. A Phase Ⅰ/Ⅱ trial evaluating everolimus for advanced HCC is currently starting to recruit patients[117] (NIH, NCT00390195). Moreover, the use of rapamycin and analogues for combination treatment together with conventional cytostatic drugs such as doxorubicin or vinblastine has been demonstrated to additively or even synergistically enhance the antineoplastic potency of the respective monotherapeutic HCC treatment with either doxorubicin or vinblastine alone[118-120].

Taken together, the *in vitro* and preclinical *in vivo* data as well as the clinical trials conducted so far show that mTOR inhibitors, including the rapamycin analogues CCI-779, RAD001 and AP23573, are promising combination agents for future cancer therapy. They are well tolerated and can produce stable disease or even substantial responses in relapsed or conventional therapy resistant solid tumours[115].

### *Proteasome inhibition*

Another interesting therapeutic approach for innovative cancer treatment is the inhibition of the 26S proteasome, which is a large protease that is present in both the nucelus and the cytoplasm of eukaryotic cells and functions as an identifier and destructor of proteins branded for destruction by the ubiquitin system. The so called ubiquitin-proteasome pathway (UPP) is the major non-lysosomal proteolytic system in eukaryotic cells and triggers degradation of proteins involved in cell cycle progression, apoptosis, nuclear factor kappaB (NF-κB) activation, and angiogenesis. UPP also degrades mutant, damaged, and misfolded proteins[121]. Since these signalling pathways are critical for cell survival and proliferation, especially in cancer cells, the inhibition of the proteasome has emerged as an attractive target for cancer therapy.

Bortezomib (Velcade™) is a proteasome inhibitor, which blocks multi-ubiquitinated protein degradation by reversibly and competitively inhibiting the active site threonine residue of the 26S proteasome[122]. Antineoplastic activity of bortezomib has already been shown in several *in vitro* and *in vivo* studies[104,123,124]. Bortezomib is the first proteasome inhibitor which has been approved for cancer therapy and is in use for the treatment of advanced multiple myeloma[121]. Based on the results of a phase Ⅱ trial on bortzezomib in the treatment of mantle cell lymphoma (ML) the FDA recently granted approval to bortezomib for the treatment of patients with ML (www.cancer.gov)[125]. Other cancers, including neuroendocrine tumours, RCC, NSCLC, or metastatic sarcomas have also been evaluated in recent phase Ⅱ clinical trials. In some of these studies a significant antineoplastic effect of monotherapy with bortezomib was observed, while in some other studies no or only marginal responses to single treatment with bortezomib were found[126-128]. However, in these cases it was recommended to investigate the role of bortezomib in combination with other antitumoural drugs. The rationale for using bortezomib in combination treatment regimes is that bortezomib's mode of action is mainly based on the inhibition of the NF-κB pathway, which has been shown to exert chemosensitizing effects when administered together with other antitumoural drugs. Combination treatment studies with encouraging results have been reported for lung cancer and lymphoma[129-131]. A phase Ⅰ/Ⅱ trial of bortezomib in patients with unresectable HCC was recently reported to result in disease stabilization in some patients and the treatment was generally well tolerated. In this study it was also suggested to especially focus on combination treatment strategies using bortezomib together with HCC-relevant cytostatics such as doxorubicin[132]. We have recently conducted an *in vitro* evaluation of bortezomib-based treatment of HCC cells. Our findings underline the suitability of bortezomib for the treatment of HCC-alone or in combination with sorafenib. In Huh-7 and Hep-G2 cells nanomolar concentrations of bortezomib induced a marked growth inhibition after three days of treatment. Moreover, the combination of bortezomib and sub-$IC_{50}$ concentrations of sorafenib resulted in additive growth inhibition in both hepatocellular Huh-7 and hepatoma Hep-G2 cells (Figure 3). Thus, our data support the idea of dual-targeting hepatocellular carcinoma cells for enhanced treatment efficacy using bortezomib as a combinatory drug. Our data strengthen the conception of multi-kinase inhibition plus bortezomib to be a promising combination for future HCC treatment, warranting further elucidation in clinical trials. Table 4 summarizes the current status of multi-kinase inhibitors and growth factor independent inhibitors for the treatment of solid tumours.

## CONCLUSION

The concept of targeted-therapies which specifically inhibit growth factor receptors and their related signalling pathways emerged to be a promising approach for the innovative and effective medical treatment of various cancers, including hepatocellular carcinoma. Thus, advanced HCC is no longer a tumour disease without specific medical treatment options. The recent findings and clinical trials clearly demonstrate that especially combination treatments inhibiting more than just one

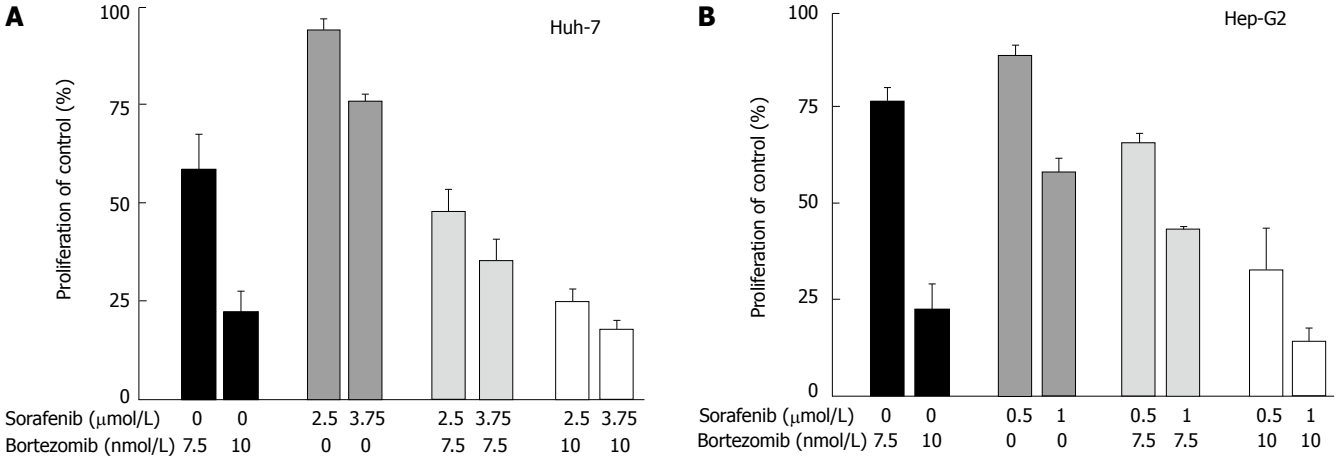


**Figure 3** Antiproliferative effects of combination treatment with bortezomib and sorafenib in hepatocelluar carcinoma cells. **A**: Huh-7 and **B**: Hep-G2 cells were treated for 72 h with sub-$IC_{50}$ concentrations of sorafenib and the proteasome inhibitor bortezomib. Combination of both agents led to additive growth inhibition both in Huh-7 as well as in Hep-G2 cells (mean ± SEM).

**Table 4 Agents of multi-kinase- and growth factor independent inhibition for the therapy of solid tumours**

| Name | Target | Mechanism | Current status |
|---|---|---|---|
| Sorafenib | c-Raf-1, B-Raf, VEGFR, PDGFR | Tyrosine kinase inhibitor | Approved for advanced RCC<br>Phase Ⅲ for advanced HCC[108]<br>Phase Ⅱ for melanoma[106], breast cancer[158] and NSCLC[159]<br>Phase Ⅰ for advanced solid tumours[160] |
| Everolimus (RAD001) | mTOR | Tyrosine kinase inhibitor | Phase Ⅱ for colorectal cancer[165]<br>Phase Ⅰ / Ⅱ for advanced HCC[117,116]<br>Phase Ⅰ for endometrial and brain tumours[111,112] |
| Temsirolimus (CCI-779) | mTOR | Tyrosine kinase inhibitor | Phase Ⅱ for metastatic breast cancer[161], advanced RCC[162], and mantle cell lymphoma[163]<br>Phase Ⅰ for advanced solid tumours (e.g. colorectal, ovarian, lung cancer)[164] |
| AP23573 | mTOR | Tyrosine kinase inhibitor | Phase Ⅱ for sarcomas of soft tissue and bone[114]<br>Phase Ⅰ for advanced solid tumours |
| Bortezomib (Velcade) | Proetasome | Proteasome inhibitor | Approved for multiple myeloma and mantle cell lymphoma[125]<br>Phase Ⅱ for colorectal cancer[166], neuroendocrine tumours[128], sarcoma[126], RCC[127], and NSCLC[129]<br>Phase Ⅰ / Ⅱ for unresectable HCC[132]<br>Phase Ⅰ for advanced solid tumours[167] |

signalling pathway will be particularly efficient, as it leaves less mechanisms of escape for the tumour cells.

In addition, there are several other promising new drugs which are currently being tested or which should be investigated in future HCC trials. In this respect combinations with drugs such as multi-kinase inhibitors are particularly intriguing. Thus in the future agents like the multi-kinase inhibitor sorafenib will likely be combined with growth factor receptor inhibitors, proteasome inhibitors, HDAC inhibitors or cytostatics as to effectively control advanced HCC. The advantage of such novel combination therapies is their higher efficacy at lowered toxicity as compared to monotherapeutic approaches. The novel combination treatments will offer new chances for drug therapy even in HCC patients with underlying cirrhosis. Fortunately, most of the new drugs can be taken orally.

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*REVIEW*

# Historical perspective of living donor liver transplantation

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## Abstract

Living donor liver transplantation (LDLT) has gone through its formative years and established as a legitimate treatment when a deceased donor liver graft is not timely or simply not available at all. Nevertheless, LDLT is characterized by its technical complexity and ethical controversy. These are the consequences of a single organ having to serve two subjects, the donor and the recipient, instantaneously. The transplant community has a common ground on assuring donor safety while achieving predictable recipient success. With this background, a reflection of the development of LDLT may be appropriate to direct future research and patient-care efforts on this life-saving treatment alternative.



**Key words:** Historical perspective; Living donor liver transplantation

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## INTRODUCTION

Living donor liver transplantation (LDLT) has been rapidly growing and evolving since its debut in 1989[1], while deceased donor liver transplantation (DDLT) had already been a standard procedure for a decade[2,3]. LDLT being the legitimate remedy for the refractory shortage of deceased donor liver grafts is characterized by its technical complexity and ethical controversies.

In 1963, Starzl described in detail three cases of DDLT. The first recipient was a 3-year-old boy with biliary atresia and died from intra-operative hemorrhage. The other two recipients were adult males with primary liver cancer. Both succumbed to pulmonary embolism 7 and 22 d after transplantation. These three cases, though ended up in hospital mortality, established the technical feasibility of liver transplantation in human[4]. Only four years later, long survivals were achieved in four DDLT recipients[5]. To become a reliable treatment modality for end-stage liver diseases of a number of etiologies, DDLT has taken two important steps: the clinical use of calcineurin inhibitor - cyclosporine A[6] and improvement of graft preservation techniques by hypothermic perfusion utilizing University of Wisconsin solution[7].

Soon after DDLT had become a standard clinical practice, it outstripped the supply of deceased donor liver grafts. The shortage of pediatric deceased donor liver grafts was even more marked. To overcome size disparity of the graft and the child recipient, reduced-size liver transplantation was devised by Bismuth in 1984[8]. Through extension of this concept, split-graft liver transplantation was then developed by Pichlmayr in 1988[9]. This enables transplanting one more recipient and circumventing graft size discrepancy in one go. The first series was reported by Broelsch in 1990[10].

## BIRTH OF LIVING DONOR LIVER TRANSPLANTATION

Experience gained from in-situ donor hepatectomy in reduced-size and split-graft DDLT paved the way for LDLT, an idea proposed by Smith as early as 1969[11]. When harvesting was performed on the living donor, much more technical ingenuity was required. The first attempt was made by Raia[1] and first success achieved by Strong of Australia[12] in July 1989. Under stringent review and auspices of the internal review board[13], the Chicago group led by Broelsch developed the first adult-to-child LDLT program[10]. Small series of adult-to-child LLDT were then reported from the United States[14] and Europe[15].

The problem of deceased donor liver graft shortage has been particularly severe in Asia[16]. In Japan, where deceased donor graft donation was non-existent[17] and liver surgery already well-developed, LDLT flourished[18,19]. For adult-to-adult LDLT (ALDLT), the left liver was used initially and was reported by the Shinshu group[20]. The left lobe used for adults was very often handicapped by the inadequate graft size. In 1993, Kyoto reported their improvisation of using the right lobe in a case of adult-to-child LDLT for a 9-year old recipient. The intention in this particular case was to avoid precarious arterial anatomy of the donor's left lobe[21]. The first case of right lobe ALDLT was performed at Queen Mary Hospital, the University of Hong Kong on May 10th 1996. *A priori*, the right liver

graft design included the middle hepatic vein (MHV). This was to address the problem of small-for-size syndrome[22]. The first series was reported shortly[23].

Donor right hepatectomy is one of the most major surgical living donor procedures. Subjecting a donor who has no medical indication for surgery to a major surgical operation with attendant risks is an ethical challenge. It was viewed by the medical community and the society with caution and skepticism[24-26]. Such donor procedure could only be partially justified by the benefit on the recipient and exhaustion of alternatives. This view is not universally accepted. Our common ground is the commitment to provide care of the highest standard to the living liver donor. Efforts for the betterment of care for the donors and yet not depriving them of the chance of saving or improving the life of their beloved recipients should worth dedication and ingenuity of the transplant community.

## DONOR SAFETY AND WELLBEING

Donor safety is central to LDLT. As the application of LDLT extended from children to adults, and from using the left liver graft to the right liver graft, the dilemma between recipient success and donor risk came to the spotlight. The reported overall complication rate of donors is around 20%, but as high as 67% in one review[27]. A unified system of complication reporting[28] may narrow this range. Not only does the complication rate vary amongst different centers, the types of complications reported also vary. The most common complications are wound infection, ileus, and bile leakage. With accumulation of experience, donor morbidity could be lower than 20%. The majority of complications are of Grade I and were wound infections. With careful attention to biliary anatomy and guidance from intraoperative cholangiography, biliary complications are avoidable[29]. While one donor mortality is too many for the transplant community, there are already 14 known donor deaths[30]. Donor right hepatectomy carries a 0.5% donor mortality rate[30]. Similarly, the causes of donor mortality also vary[31]. A widely publicized case is a male donor in New York who succumbed to gas gangrene of *Clostridium perfringens* 3 d after donor right hepatectomy[32]. A hypertensive lady in Japan died from liver failure after right liver donation with a residual left liver with nonalcoholic steatohepatitis 28% of the total liver volume[33]. Fatal pulmonary embolism also occurred in a left liver donor[34]. A donor mother with a history of substance abuse also died from drug overdose 2 mo after donation to her 3 year-old son[35]. In other words, achieving a five-year recipient survival of 80%, it takes one donor life to save 160 recipients. Less tangible is the quality of life changes of the donor in comparison to the predonation state. The long-term biological consequences of donor hepatectomy are not fully known. Nevertheless, there are demonstrable drops in white cell count, platelet counts and elevation of liver transaminases even two years after right liver donation[36]. Quantification of such is mandatory in defining the field strength of LDLT. Detail of the holistic care of living liver donors deserves elaboration in a separate synopsis.

As agreed by the liver transplant community, living liver donors should be of good health[30] and the donor operation performed by experienced centers[37]. There should be no compromise of accepting potential donors with suboptimal physical and mental health. This is the only way to maintain or decrease donor mortality and morbidity.

## RECIPIENT SHORT-TERM OUTCOMES

### *Graft size*

Recipient survival is dependent on adequate graft size in relation to recipient body size[38]. Pathophysiology of the small-for-size graft and small-for-size syndrome is then defined[39]. Features include hepatocyte ballooning, steatosis, centrilobular necrosis, and parenchymal cholestasis. Pre-existing portal hypertension of the recipient increases the size requirement of graft[40].

Anecdotal success of using a very small graft for ALDLT 25% of the estimated standard liver weight[41] and even 20% with portosystemic shunting[42] had been reported. The paradigm shift from the left liver to the right liver enables adult recipients to undergo LDLT[43]. With technical maturity, 35% of the estimated standard liver weight remains the minimum requirement of a graft for predictable recipient success[44]. Portal hyperperfusion[45] and portal hypertension[46] are now conceived as possible mechanisms conducive to damage of small-for-size grafts. A battery of techniques for alleviation of portal venous flow was described. This includes superior mesenteric vein to mesocaval shunt[47], hemiportocaval shunting[48], inflow modulation by splenic artery ligation[49]. With portosystemic shunting using a saphenous vein interpositional graft between the right portal vein and right hepatic vein stump, a left lobe 20% of the estimated standard liver mass had been transplanted successfully in one patient[42]. Pharmacological manipulation is on the horizon as well[50].

More basic to these is the accurate assessment of standard liver volume of the recipient and thus the minimum graft size requirements. There have been a number of formulae developed from the west[51,52] and one from Japan[53]. A formula derived from Chinese and for application in Chinese which is also gender dependent has been developed and for validation[54].

### *Middle hepatic vein*

Center to the controversy of right lobe ALDLT is inclusion of the MHV or otherwise. Deleterious effects of no drainage to the segments 5 and 8 include severe venous congestion and necrosis of these segments[55]. Surgical decision of not including the MHV includes demonstration of collaterals between segment 5 and 8 tributaries and the right hepatic vein[56]. Kyoto University devised an algorithm which includes the MHV when the graft is MHV dominant, or the graft to recipient weight ratio less than 1%, and in all cases, remnant left lobe larger than 35%[43]. Chang Gung Memorial Hospital includes the MHV when the graft to estimated standard liver volume is 50% or less, or when segment 5 and 8 hepatic veins are large and the right hepatic vein small[57]. Tokyo University ingeniously observed congestion of segments 5 and 8 of the graft after temporary clamping of the right hepatic artery before determining venous interpositional grafting[58].

We include the MHV in all right liver grafts for simplicity and familiarity of the technique[59]. Irrespective of the venous drainage pattern of segment 4 of the remnant left liver, the segment 4b hepatic vein is preserved. Utmost care is needed for its preservation when it drains into the MHV[60]. The outflow capacity is guaranteed by venoplasty on the back-table of the MHV and right hepatic vein into a single cuff[61]. The venoplasty is further marked by a more expedient hepatic vein to inferior vena cava anastomosis and higher outflow capacity of the right liver graft[62].

In summary, adequate graft size and quality, excellent venous outflow, and moderate portal inflow are keys to success of ALDLT[63].

### *High urgency LDLT*

ALDLT under high urgency was impetus to development of liver transplant in our center[23]. Early experience of a number of centers showed inferior surgical outcomes of ALDLT in the high urgency situation[64-66]. With accumulation of experience and right liver graft incorporating the MHV, surgical outcomes of ALDLT is not compromised[67]. We also showed that ALDLT improves the survival of potential recipients[68]. The question of when a patient becomes too sick for liver transplantation is to be answered[69]. To justify ALDLT, good recipient outcome and acceptable donor morbidity, and voluntarism of the donor are the least that could be expected. The Live Organ Donor Consensus Group has largely supported this viewpoint[70]. The Model for End-Stage Liver Disease score has been validated as a factor predictive of recipient short-term survival in DDLT[71]. Data from ALDLT in North America[72] and Europe[73], however, do not support this view. Outside Asia, ALDLT is gradually considered a standard treatment for acute liver failure.

### *Biliary reconstruction*

Biliary complication justifies itself the Achilles' heel of DDLT and is even more convincing in ALDLT[67]. Hepaticojejunostomy and duct-to-duct anastomosis have no substantial difference in the incidence of biliary complications. Nonetheless, duct-to-duct anastomosis reduces the operating time and avoids contamination of the operation field, expedites return of bowel functions, and avoids internal herniation of bowel loops. It also allows subsequent intervention by endoscopic retrograde cholangio pancreatography. In some centers, duct-to-duct anastomosis is stented to minimize the chance of stensosis and leakage. Whether the stent plays a role in the postoperative period, or in facilitating anastomosis, or both, has not been validated. Furthermore, whether continuous or interrupted sutures makes a difference is unknown. A study on DDLT which showed no difference[74] may not be applicable to ALDLT. Randomized controlled trials of recipients allocated to both arms may answer these questions.

## RECIPIENT LONG-TERM OUTCOMES

### *Hepatocellular carcinoma*

Early efforts of transplanting patients with advanced unresectable primary liver cancers were tempered by invariable relapse of malignancy[75]. Further work of the same group established the correlation between poor prognosis and high pathological tumor-node-metastasis staging[76]. Vascular invasion by tumor is the single most important factor in treatment failure of ALDLT for unresectable small hepatocellular carcinoma (HCC). Major vascular invasions though apparent for large tumors, may not be so for the small ones. Now called the Milan Criteria[77] and the University College of San Francisco Criteria (UCSF)[78], the tumor size and number are used as surrogate parameters for likelihood of vascular invasion. The Milan criteria are based on pretreatment imaging, whereas the UCSF criteria on liver explant histopathology. Accuracy of preoperative imaging in staging is inadequate[79]. The tendency is toward underestimation of tumor load. Even in studies with good image to histopathology correlation, underestimation is common. Tumor grade[80] and tumor size[81,82] are predictors of vascular invasion. Tumor size itself is also a predictor of tumor grade[81,82].

In our own series of ALDLT for HCC, there is a tendency of a higher recurrence rate compared with DDLT. It is postulated that the higher regeneration rate and reperfusion injury of small grafts in ALDLT provides an environment favorable for HCC cell implantation and growth in the graft[83]. It is also possible that in ALDLT, for preservation of the inferior vena cava, more liver manipulation is required leading to tumor compression and cancer cell dissemination. However, it is not unlikely that patients who have received DDLT are the self-selected patients because only candidates with slowly growing HCC who could wait for deceased donor liver grafts could receive the transplantation as the cancer cells are less aggressive. In fact, fast-tracking ALDLT for HCC had a higher recurrence rate[84]. Further studies on patient selection criteria and innovation of surgical technique are required to improve the long-term outcome of ALDLT for HCC. A recent series from Korea, nonetheless, has comparable results as DDLT[85].

In a series of 316 recipients with HCC who underwent ALDLT in Japan, the patient and recurrence-free survival rates were significantly worse if the Milan's criteria were not met. However, within this series, 171 (54.1%) of the recipients did not fulfill the Milan's criteria, and 176 were staged IVa. The alpha-fetoprotein level, tumor size, vascular invasion, and bilobar distribution were independent risk factors for HCC recurrence. The grade of histological differentiation of HCC showed close correlation with tumor characteristics and recurrence. Multifocal HCC verified by histopathology after transplantation with no recurrence was reported[86]. A policy of extended indication beyond the Milan and UCSF criteria is being validated[87].

The first use of sirolimus in liver transplantation was in patients with liver cancer, using the reported antitumor effects of the drug[88]. Subsequent work has shown efficacy of the drug in the inhibition of hepatocellular tumor cell lines[89,90]. Recipients transplanted for HCC and received sirolimus and low dose tacrolimus survived longer[91].

### *Sequelae of long-term immunosuppression*

Calcineurin inhibitors greatly improve graft and recipient

**Table 1 Landmark publications of liver transplantation in chronological order**

| Author | Discovery and application | Year of publication |
|---|---|---|
| Starzl[4] | First attempts of DDLT in human (1963) | 1963 |
| Starzl[5] | First long survival DDLT recipients (1967-1968) | 1968 |
| Smith[11] | Conceptualization of LDLT (1969) | 1969 |
| Calne[6] | Cyclosporin A used in solid organ transplantation | 1979 |
| Bismuth[8] | First adult-to-child reduced-size DDLT (1981) | 1984 |
| Pichlmayr[9] | Split-graft DDLT for 2 recipients (1988) | 1988 |
| Belzer[7] | Clinical use of University of Wisconsin Solution | 1990 |
| Raia[1] | First attempt of LDLT (December 1988) | 1989 |
| Strong[12] | First successful LDLT from adult to child (July 1989) | 1990 |
| Yamaoka[21] | First report of right liver graft from adult to child (1992) | 1994 |
| Hashikura[20] | First successful left liver adult-to-adult LDLT (November 1993) | 1994 |
| Lo[22] | First right liver adult-to-adult LDLT using right liver (May 1996) | 1997 |

survivals. The lowest permissible serum drug level is employed to minimize the side effects. Nonetheless, two undesirable results still occur in recipients, i.e. renal impairment and tendency toward diabetes. A trend toward steroid-sparing immunosuppression seems workable[92]. The added benefit of the potential antineoplastic property of sirolimus makes it very attractive for recipients with renal impairment transplanted for resectable HCC[93].

Prompted by development of immune tolerance of noncompliant liver recipient after cessation of immunosuppressant therapy, weaning programs were incorporated into a long-term strategy of liver transplant programs. Drug-free tolerance was observed more frequently in humans after transplantation of the liver than of any other organs. Clinical application of cyclosporine[6] and then tacrolimus[94] dramatically prevented acute rejection of transplanted organs. However, drug-free tolerance became rare with the dominance of multiagent prophylactic immunosuppression[95].

Development of tolerance to the graft obviates the use of immunosuppressant with the side effects. The equivalence of solid organ and bone marrow transplantation is substantiated by documentation of systemic microchimerism[96]. Liver cells were identified in distant organs and host cells were also identified in liver grafts. The mirror image of solid organ and bone marrow transplantation envisioned by Starzl brings to light the practicality of long-term donor specific tolerance. The liver as a privileged graft[97] is a constant source of donor leukocyte, thus facilitates the process of clonal exhaustion-deletion especially in the early post-transplant phase under low immunosuppression pretransplant by antilymphocyte globulin. The concept of prope tolerance is also proposed as a means to a similar condition at a lesser degree[98]. Utilizing a powerful lymphocyte-depleting antibody, Campath 1H, half-dose cyclosporine monotherapy became workable. During a window of opportunity for immunologic engagement (WOFIE), it is hypothesized that there is engagement of donor and recipient marrow cells. Not until the availability of transgenic xenografts, effective and specific immunosuppression remains the practical way to graft maintenance. Nonetheless, continual assessment of the risk of chronic subclinical rejection is necessary[99].

## CONCLUSION

At a minimum, recipient success is high and donor risk low. This brings donor and recipient issues into a close relationship. Conceptually, it would be inappropriate to accept a higher risk for the donor simply because of the improvement of recipient outcome. It is nonetheless our common goal to improve the standard of recipient and donor operations. What the public should change is, however, the better acceptance of ALDLT in the face of better safety and success, while the effort to make more deceased donor grafts available is never be forgotten.

Now we have near perfect graft harvesting and implantation techniques. Excluding patients with prohibitive conditions, e.g. uncontrolled sepsis and poor cardiac conditions, the short-term success is predictable. We still require selecting patients with a low recurrence rate of HCC and hepatitis C after transplantation. A lower biliary complication rate is welcome and could only be reduced by better preservation of biliary vasculature on the donor and the recipient and careful anastomotic techniques.

Donor safety and recipient success are inseparable. While donor mortality is a reality, it is by lowering donor mortality and improving recipient survival the justification of LDLT becomes stronger.

Although the major interest of the liver transplant community was in ALDLT in the last decade, the success of ALDLT has been a result of the ground works laid since the sixties. Key publications documenting the major achievements in liver transplantation leading to the ever improving results of ALDLT are listed in chronological order in Table 1.

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*TOPIC HIGHLIGHT*

**David R Gretch, MD, PhD,** *Series Editor*

# Role of peroxisome proliferators-activated receptors in the pathogenesis and treatment of nonalcoholic fatty liver disease

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## Abstract

Nonalcoholic fatty liver disease (NAFLD) is highly prevalent and can result in nonalcoholic steatohepatitis (NASH) and progressive liver disease including cirrhosis and hepatocellular carcinoma. A growing body of literature implicates the peroxisome proliferators-activated receptors (PPARs) in the pathogenesis and treatment of NAFLD. These nuclear hormone receptors impact on hepatic triglyceride accumulation and insulin resistance. The aim of this review is to describe the data linking PPARα and PPARγ to NAFLD/NASH and to discuss the use of PPAR ligands for the treatment of NASH.



**Key words:** Non-alcoholic fatty liver disease; Peroxisome proliferators-activated receptors; Insulin resistance; Metabolic syndrome; Pharmacologic ligands

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## BACKGROUND ON NAFLD/NASH

An estimated 30% of adults and 10% of children and adolescents in the United States have nonalcoholic fatty liver disease (NAFLD), defined as liver fat content exceeding 5% (Figure 1)[1-3]. Non-alcoholic fatty liver disease is associated with obesity, non-insulin dependent diabetes, and hypertriglyceridemia and represents the hepatic manifestation of the metabolic syndrome[4]. A subset of persons with NAFLD progresses to nonalcoholic steatohepatitis (NASH), consisting of hepatic steatosis accompanied by inflammation and fibrosis (Figure 1)[5]. Nonalcoholic steatohepatitis affects approximately 3% of the lean population and 19% of obese persons, making it the most prevalent cause of chronic liver disease in the country[6]. Moreover, NASH represents a progressive form of liver disease. Cirrhosis developed in 5% of patients with NASH in a community-based cohort and 20% of NASH patients in a referral population[7,8]. Nonalcoholic steatohepatitis accounts for up to 75% of cases of cryptogenic cirrhosis and patients with NASH and cirrhosis are at risk for hepatocellular carcinoma[9,10].

The pathogenesis of NASH is often conceptualized as a two-step process, consisting of hepatic triglyceride accumulation, followed by the development of oxidative stress and cytokine expression leading to steatohepatitis[11]. Multiple metabolic processes can result in hepatocellular triglyceride accumulation including: (1) Excess dietary intake. Dietary triglycerides are delivered to the liver in the form of chylomicrons. In addition, dietary calories stored in adipose tissue as fat represent a source of fatty acids and triglycerides that can be delivered to the liver in the form of lipoprotein particles and free fatty acids. (2) Increased rates of lipogenesis resulting from the *de novo* synthesis of fatty acids and triglycerides in the liver. (3) Decreased rates of β-oxidation of fatty acids in the liver. (4) Decreased rates of export of cholesterol esters and triglycerides from the liver as very low density lipoprotein (VLDL)[12]. As shown in Figure 2, the PPARs impact on multiple processes involved in lipid trafficking and metabolism.

Insulin resistance and hyperinsulinemia seem to be central to the development of NAFLD. Insulin resistance is associated with increased lipolysis and reduced postprandial uptake and storage of fatty acids in adipose tissue, leading to increased fatty acid flux to the liver[13]. In turn, increased liver fat content contributes to hepatic insulin resistance[14]. Hyperinsulinemia induces sterol regulatory element-binding protein-1c (SREBP-1c) expression and hyperglycemia activates carbohydrate response element binding protein (ChREBP), both of which increase hepatic fatty acid synthesis[15].

## THE PPARS

PPARs play a key role in modulating hepatic triglyceride

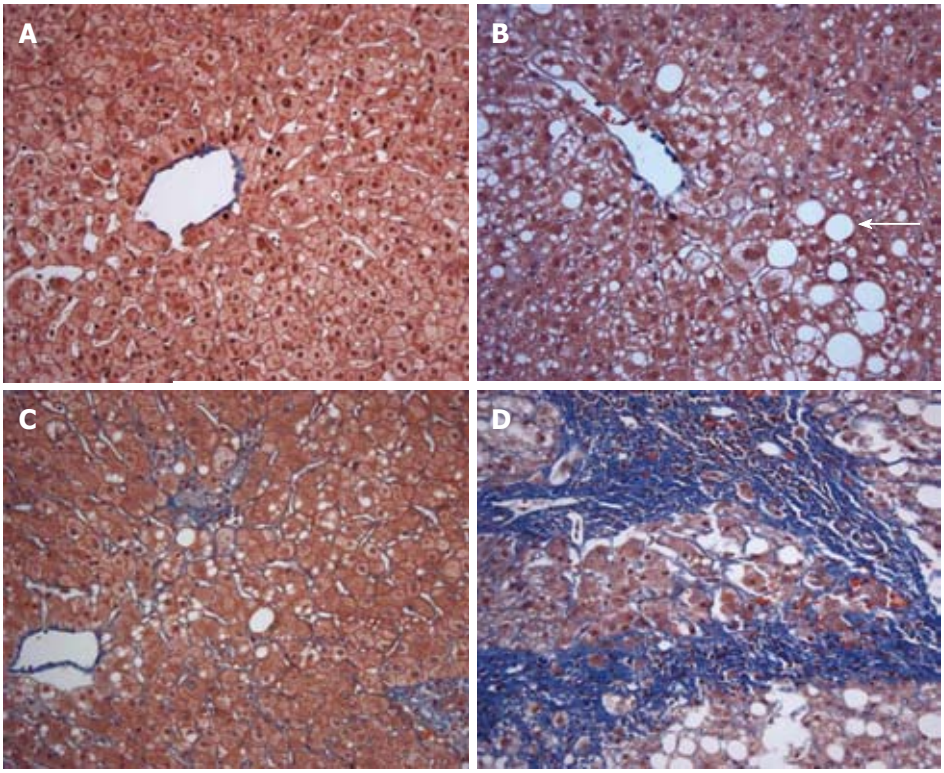


**Figure 1** Liver histology ranging from normal liver to steatohepatitis with fibrosis. **A**: Normal liver. Cytoplasmic fat globules are absent in hepatocytes and there is no fibrosis in this trichrome stained specimen (× 20); **B**: Steatosis without steatohepatitis. Moderate cytoplasmic fat infiltration (arrow) is present without fibrosis (× 20); **C**: Steatohepatitis with minimal fibrosis. There is focal hepatocyte ballooning, inflammation, and minimal fibrosis (accentuated in blue by trichrome stain) (× 20); **D**: Steatohepatitis with fibrosis. There is nodular scarring in this fat laden liver with advanced fibrosis depicted in blue by trichrome stain (× 20).

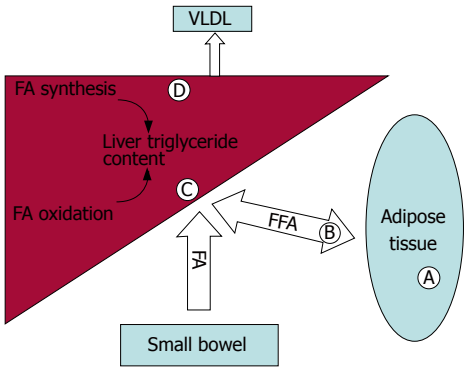


**Figure 2** Mechanisms by which PPARs and their ligands can modulate triglyceride accumulation are highlighted by letters in the figure. A: PPARγ increases expression of genes associated with fatty acid uptake and triglyceride storage in adipocytes. Release of adiponectin from adipocytes improves insulin sensitivity and activates PPARα; B: PPARγ increases lipoprotein lipase expression, liberating circulating fatty acids from lipoproteins for import into adipocytes; C: PPARα activity up regulates β-oxidation of fatty acids in the liver; D: PPARα and TZDs upregulate stearoyl-CoA desaturase-1, a necessary enzyme for VLDL synthesis and export, and TZDs increase arachidonic acid content in triglycerides, which is associated with increased insulin sensitivity.

accumulation. PPARα regulates fatty acid β-oxidation. PPARγ increases insulin sensitivity as well as regulating triglyceride storage in adipose tissue. Fat labeling studies indicated that the majority of hepatic triglycerides originate from adipose tissue as non-esterified fatty acids[16].

PPARs are part of the nuclear receptor superfamily[17]. There are three isotypes in mammals designated PPARα [NR1C1], PPARδ [NR1C2] and PPARγ [NR1C3][18]. PPARα is activated by ligands termed peroxisome proliferators, which were named for their effects on peroxisomes in rodent livers[19,20]. Lipids are natural PPAR ligands, leading to regulation of lipid metabolism and fuel partitioning[17]. PPARs form a heterodimer with the retinoid X receptor (RXR). The PPAR:RXR heterodimer, when bound to a ligand, changes conformation and binds to DNA at PPAR response elements, resulting in gene transcription[21,22].

## PPARα AND NAFLD

PPARα is expressed in the liver and other metabolically active tissues including striated muscle, kidney and pancreas[23,24]. Many of the genes encoding enzymes involved in the mitochondrial and peroxisomal fatty acid β-oxidation pathways are regulated by PPARα. In particular, the acyl-CoA synthetase, the carnitine palmitoyl transferase I, the very long-chain acyl-CoA dehydrogenase and the tri-functional protein genes encoding enzymes in the mitochondrial fatty acid β-oxidation pathway are induced by peroxisome proliferators that activate PPARα[25-28]. Similarly, the acyl-CoA synthetase, the straight-chain acyl-CoA oxidase, the L-bifunctional protein and the 3-ketoacyl-CoA thiolase genes encoding enzymes in the peroxisomal fatty acid β-oxidation pathway are induced by peroxisome proliferators that active PPARα[26,27,29,30]. Loss of expression of the PPARα gene in mice results in hepatic steatosis under conditions of increased fatty acid metabolism in the liver such as fasting or a high fat diet[31,32]. Administration of a potent PPAR agonist decreases hepatic steatosis in mice receiving a methionine and choline deficient diet[33]. These observations indicate that under conditions of increased hepatic fatty acid influx or decreased hepatic fatty acid efflux, PPARα activation prevents the accumulation of triglycerides by increasing the rate of fatty acid catabolism.

Additional factors appear to interact with PPARα to regulate hepatic triglyceride content. These include adiponectin, which is an adipocyte produced peptide

hormone that limits fat accumulation in the liver by a number of mechanisms including activation of PPARα to increase hepatic fatty acid oxidation[34]. In cell culture models, treatment with adiponectin resulted in increased activity of PPARα target genes such as acyl-CoA oxidase, carnitine palmitoyl transferase- I , and fatty acid binding protein[35]. PPARα ligands can increase steaoryl-CoA desaturase-1 (SCD1) activity, which is necessary for VLDL secretion[36]. A PPAR response element was found in the SCD1 promoter[37]. Adiponectin is upregulated by PPARγ, providing a connection between the two isotypes[38].

Most of the data regarding PPARα and hepatic lipid homeostasis comes from mouse models. However, there are important differences in PPARα activity between rodents and humans. PPARα DNA binding activity and PPARα expression in human hepatocytes is less than 10-fold that observed in mice[39,40]. Certain PPAR response elements, such as the acyl CoA oxidase gene, do not respond to PPAR ligands in humans as they do in rodent models[41]. Finally, PPARα activation in rodent models resulted in peroxisomal proliferation, hepatomegaly, and hepatocellular carcinoma[39,42,43], whereas similar changes were not observed in humans[44,45]. Further research is needed to determine the relative importance of PPARα in regulating hepatic triglyceride metabolism in humans.

## PPARα AS A TARGET FOR THE TREATMENT OF NAFLD

Fibric acid derivatives, which are available for use in humans as lipid lowering agents, serve as PPARα activators[46,47]. In a mouse model of fatty liver disease, fenofibrate treatment improved steatosis and increased expression of genes involved in fatty acid metabolism[48]. Trials with fibrates in humans have yielded mixed results. A study involving potential living liver donors with steatosis showed that a combination of diet, exercise, and benzafibrate significantly reduced steatosis and resulted in normalization of alanine aminotransferase levels[49]. However, it was not clear whether the therapeutic benefit was related to benzafibrate or to a 1000 kilocalorie/day diet and a 600 kilocalorie/day exercise regimen. In addition to being a PPARα ligand, benzofibrate activates PPARγ and improves insulin sensitivity in animal models[46,47], an effect not seen with fenofibrate[50]. Another study demonstrated that 42% of 62 patients with NAFLD had biochemical and ultrasound improvement on fenofibrate, but histologic data were not collected[51]. A small controlled study of gemfibrozil *versus* placebo for four weeks found improved aminotransferase levels with the use of gemfibrozil in patients with NAFLD[52]. These studies are in contrast to a another small series, which demonstrated no change in aminotransferases and no histologic improvement after one year of clofibrate therapy for NAFLD[53].

Omega-3 polyunsaturated fatty acids (PUFA) present in fish oil, and their metabolites, provide another source of PPARα ligands. Omega-3 PUFA also inhibit lipogenesis by antagonizing activation of LXR[54,55], thus reducing expression of SREBP-1c[56], which results in the down regulation of key enzymes involved in hepatic lipid biosynthesis. In mouse models, omega-3 PUFA supplementation was associated with improvement in hepatic steatosis and insulin sensitivity, as well as lower fasting free fatty acid concentrations and lower serum triglyceride levels[57,58]. Two human studies reported a decline in serum aminotransferase levels and improvement in ultrasound features of fatty liver with omega-3 PUFA supplementation[59,60]. However, no histologic data were provided. Omega-3 PUFA supplementation also reduces serum triglyceride levels in the fasting and postprandial state[61-63], but was not found to improve insulin sensitivity in humans[62,64,65].

## PPARγ AND NAFLD

PPARγ is expressed in high levels in adipose tissue[66] and plays a role in increasing insulin sensitivity as well as in promoting fatty acid uptake into adipocytes and adipocyte differentiation. The net effect of these processes is to increase triglyceride storage in adipocytes, reducing delivery of fatty acids to the liver. Patients with dominant negative mutations in PPARγ have NAFLD and the metabolic syndrome while lacking adipose tissue suggesting increased triglyceride delivery to the liver[67]. PPARγ is present in the liver to a lesser degree than in adipose tissue. Liver-specific PPARγ deficient mice are protected against the development of steatosis suggesting a role for hepatic PPARγ in liver triglyceride accumulation[68,69].

Insulin resistance is integral to the development of NAFLD, leading to increased fatty acid flux to the liver and increased hepatic fatty acid synthesis[13,15]. PPARγ increases insulin sensitivity by upregulating GLUT4, an insulin dependent glucose transporter in adipose tissue and striated muscle[70], and inducing expression of the c-Cbl associated protein, which is involved in insulin signaling[71]. Additionally, in mouse models of insulin resistance, PPARγ activation attenuated induction of supressor of cytokine signaling 3 (SOCS3), which is involved in the development of insulin resistance[72].

PPARγ also promotes adipocyte differentiation and expression of proteins in adipocytes involved in fatty acid uptake[17,73], fatty acid transport[74,75] and fatty acid synthesis[76]. Differentiation of preadipocytes to adipocytes requires transcription factors including the CCAT-enhancer-binding proteins (C/EBPs) and the adipocyte differentiation and determination factor (ADD)-1/SREBP-1[77-80]. C/EBP plays an important role in inducing and maintaining PPARγ expression in adipogenesis[81,82]. ADD-1/SREBP-1 is strongly adipogenic, is enhanced by PPARγ, and results in the expression of lipogenic genes including fatty acid synthase[80]. These transcription factors guide the cell through proliferation, clonal expansion, growth arrest, and eventually adipocyte specific genes are activated resulting in lipid accumulation[82]. PPARγ also increases expression of lipoprotein lipase, an enzyme that serves to partition fat to adipocytes, limiting fatty acid flux to the liver. Similar to PPARα, PPARγ ligands upregulate SCD1 activity, which promotes VLDL secretion. Thiazolidinediones (TZDs), ligands for PPARγ have also been shown to increase arachidonic acid content in triglycerides through SCD1, which has been associated with increased

insulin sensitivity[83]. Other effects of PPARγ include induction of uncoupling protein-2, which might decrease hepatic triglyceride accumulation by increasing energy expenditure[84]. PPARγ expression also might reduce hepatic inflammation by decreasing expression of proinflammatory cytokines, such as TNFα[85].

## PPARγ AS A TARGET FOR THE TREATMENT OF NAFLD

TZDs are PPARγ agonists, which improve glycemic control in patients with type 2 diabetes mellitus by increasing insulin sensitivity[86]. The TZD-mediated increase in insulin sensitivity was demonstrated in adipose tissue, the liver, and skeletal muscle[87,88]. TZD therapy increases adiponectin levels, which are associated with improved insulin sensitivity[89]. Furthermore, adiponectin impacts on hepatic fat accumulation by enhancing fatty acid oxidation in muscle, and by activating PPARα to increase fatty acid oxidation in the liver[34].

Thiazolidinediones also increase expression of AMP-activated protein kinase[88,90]. This protein kinase increases fatty acid oxidation as well as decreasing lipogenesis[91,92]. The reduction in lipogenesis is mediated through phosphorylation and inhibition of acetyl-CoA carboxylase, which decreases malonyl CoA formation and down regulates SREBP and the carbohydrate response element binding protein (ChREBP)[93]. Finally, TZDs have anti-inflammatory and anti-fibrotic properties that might be beneficial in NASH. Serum high-sensitivity CRP, IL-6 and IL-18 levels were significantly reduced in patients on TZD therapy[94,95] and the TZD pioglitazone reduced activation of hepatic stellate cells in an animal model[96]. Increased adiponectin may also contribute to the anti-inflammatory effects of TZD therapy. Adiponectin was shown to block TNFα activation of inflammatory genes in endothelial cells[97], decrease macrophage growth and function[98-100], and increase release of the anti-inflammatory cytokines IL-10 and IL-1RA with a concomitant decrease in interferon-γ production[100].

Studies of the TZDs rosiglitazone and pioglitazone demonstrated reduction in aminotransferase levels and improvement in liver histology in patients with NASH[87,101-106]. One study that compared pioglitazone plus vitamin E to vitamin E alone for the treatment of NASH found significant improvement in steatosis, hepatocellular ballooning, and pericellular fibrosis in the combination therapy arm, but not in patients treated with vitamin E alone[103]. In a study of pioglitazone plus diet versus placebo plus diet in patients with biopsy proven NASH and insulin resistance, pioglitazone therapy was associated with a significant reduction in mean serum aminotransferase levels and improved glycemic control[107]. There were significant improvements in hepatic insulin resistance as well as histologic parameters including hepatic steatosis, ballooning, and inflammation, although not fibrosis with six months of treatment. Further evaluation of the efficacy and the cardiovascular risk of TZD therapy[108] is needed before this class of medications is routinely prescribed for the treatment of NASH.

## CONCLUSION

The nuclear hormone receptors PPARα and PPARγ appear to play an important role in modulating hepatic triglyceride accumulation, the primary process in the development of NAFLD. PPARα activity reduces liver fat by increasing β-oxidation of fatty acids and PPARγ increases insulin sensitivity as well as reducing fatty acid flux to the liver. PPAR ligands show promise in the treatment of NAFLD, although further human studies are needed to define the therapeutic role of these agents.

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*BASIC RESEARCH*

# Effect of notoginsenoside R1 on hepatic microcirculation disturbance induced by gut ischemia and reperfusion

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## Abstract

**AIM:** To assess the effect of notoginsenoside R1 on hepatic microcirculatory disturbance induced by gut ischemia/reperfusion (I/R) in mice.

**METHODS:** The superior mesenteric artery (SMA) of C57/BL mice was ligated for 15 min to induce gut ischemia followed by 30-min reperfusion. In another set of experiments, R1 was continuously infused (10 mg/kg per hour) from 10 min before I/R until the end of the investigation to study the influence of R1 on hepatic microcirculatory disturbance induced by gut I/R. Hepatic microcirculation was observed by inverted microscopy, and the vascular diameter, red blood cell (RBC) velocity and sinusoid perfusion were estimated. Leukocyte rolling and adhesion were observed under a laser confocal microscope. Thirty and 60 min after reperfusion, lactate dehydrogenase (LDH), alanine aminotransferase (ALT) and aspartate transaminase (AST) in peripheral blood were determined. The expression of adhesion molecules CD11b/CD18 in neutrophils and tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6) and monocyte chemotactic protein-1 (MCP-1) in plasma were evaluated by flow cytometry. E-selectin and intercellular adhesion molecule-1 (ICAM-1) in hepatic tissue were examined by immunofluorescence.

**RESULTS:** After gut I/R, the diameters of terminal portal venules and central veins, RBC velocity and the number of perfused sinusoids were decreased, while the leukocyte rolling and adhesion, the expression of E-selectin in hepatic vessels and CD18 in neutrophils, IL-6, MCP-1, LDH, ALT and AST were increased. R1 treatment attenuated these alterations except for IL-6 and MCP-1.

**CONCLUSION:** R1 prevents I/R-induced hepatic microcirculation disturbance and hepatocyte injury. The effect of R1 is related to its inhibition of leukocyte rolling and adhesion by inhibiting the expression of E-selectin in endothelium and CD18 in neutrophils.



**Key words:** Ischemia/reperfusion; Notoginsenoside R1; Leukocytes adhesion; E-selectin; Hepatic injury

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## INTRODUCTION

It is well recognized that gut ischemia/reperfusion (I/R) induces injury of distant organs, such as liver and lung[1-3]. A rate-limiting step in the pathogenesis of I/R injury of liver and other organs is the recruitment of leukocytes to vascular endothelium[2,4,5]. Oxygen free radicals produced by gut I/R activate nuclear factor kappa-B (NF-κB)[6,7], initiate expression of selectin and adhesion molecules[8-10], and elicit release of proinflammatory mediators like tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6)[10]. The expression of L-selectin on leukocytes and E-selectin on endothelial cells induces the rolling of leukocytes along the vascular endothelium[11,12], which further promotes the expression of adhesion molecules CD11b, CD18 on

leukocytes and intercellular adhesion molecule-1 (ICAM-1) on endothelial cells, resulting in adhesion of leukocytes to vascular endothelial cells[2,4,13,14]. In addition, proinflammatory mediators produced by I/R enhance the expression of selectins and adhesion molecules, and aggravate the rolling and adhesion of leukocytes[8,15]. The adhesion of leukocytes to the vascular endothelium results in release of oxygen free radicals and proteinase, thus inducing hepatic injury[16]. In line with these findings, studies indicate that inhibition of the expression of selectins and adhesion molecules on leukocytes and endothelial cells ameliorates the adhesion of leukocytes to the hepatic vascular endothelium after gut I/R and attenuates hepatic microcirculation disturbance and injury[2,4,17].

*Panax notoginseng* (PN) is the dried root of *Panax notoginseng* (Araliaceae), a Chinese herb medicine widely used in China, Korea, Japan and other Asian countries in the treatment of microcirculatory disturbance-related diseases, such as cardiovascular disease, cerebral vascular diseases and liver dysfunction[18,19]. PN contains more than 30 different types of saponin, of which ginsenoside Rg1 (Rg1), ginsenoside Rb1 (Rb1) and notoginsenoside R1 (R1) are the eminent members[20]. Previous studies have proved that *Panax notoginseng saponins* (PNS) improve I/R-induced hepatic microcirculation disturbance[19], inhibit platelet aggregation and adhesion molecule expression, and improve vascular endothelium function[21]. It was also reported that PNS inhibit adhesion of leukocytes to rat mesentery venules and expression of neutrophil adhesion molecules CD11b and CD18 induced by lipopolysaccharide (LPS)[22]. The expression of LPS-induced vascular endothelial TNF-α is inhibited by R1 by inhibiting degradation of the inhibitor kappa-B (I-κB)[23]. It has been shown that cardiotonic pills (CP, a traditional Chinese medicine containing PN, *salvia miltiorrhiza* and *borneol*) can inhibit adhesion of leukocytes to the hepatic vascular endothelium in rats induced by gut I/R and chronical ethanol feed, and blunt the concentration increment of peripheral blood alanine aminotransferase (ALT), TNF-α and LPS[24]. However, whether R1 improves gut I/R-induced hepatic microcirculation disturbance has not yet been reported, although it is highly anticipated as a major component of PNS, and the structure of R1 has been identified (Figure 1). Therefore, by virtue of intravital microscopy the present study explored the dynamic effects of R1 on a mouse hepatic microcirculation disturbance model induced by gut I/R, especially on leukocyte rolling and adhesion on the vascular endothelium. The expression of E-selectin and ICAM-1 was also determined by immunofluorescent technique. The expression of neutrophil adhesion molecules CD11b and CD18, and the concentration of proinflammatory mediators such as TNF-α, IL-6 and monocyte chemotactic protein-1 (MCP-1) was measured by flow cytometry. The concentrations of lactate dehydrogenase (LDH), ALT and aspartate aminotransferase (AST) were also determined.

## MATERIALS AND METHODS

### Notoginsenoside and reagents

R1 (purity > 98%) was supplied by Tianjin Talsy Group (Tianjin, China). Other reagents used in experiments were as follows: rhodamine 6G (purity > 99.0%, Lot No.2350994, Fluka Co., Switzerland), FITC-rat anti-mouse CD18 monocolonal antibody (Lot No.553293, BD Biosciences PharMingen, USA), FITC-rat anti-mouse CD11b monoclonal antibody (Lot No.557396, BD Biosciences PharMingen, USA), goat polyclonal antibody against mouse E-selectin (M-20) (sc-6939, Santa Cruz Biotechnology, Inc. USA), goat polyclonal antibody against mouse ICAM-1 (M-19) (sc-1511, Santa Cruz Biotechnology, Inc. USA), rhodamine conjugated rabbit anti-goat lgG-R (Lot No.B1006, Santa Cruz Biotechnology, Inc. USA), Hoechst33342 (Lot No.6538, Santa Cruz Biotechnology, Inc. USA), mouse MCP-1 flex set (Lot No.558342, BD Biosciences, USA), mouse TNF flex set (Lot No.558299, BD Biosciences, USA), mouse IL-6 flex set (Lot No.558301, BD Biosciences, USA).

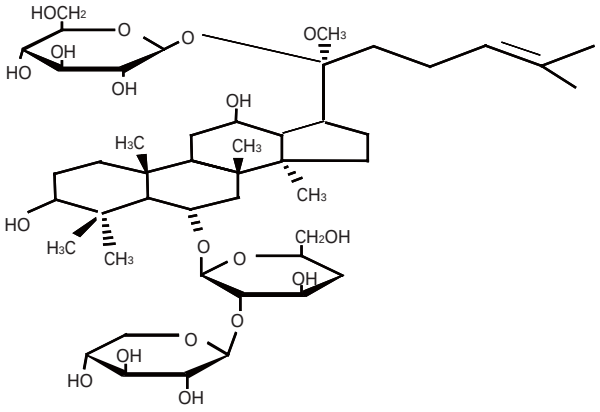


**Figure 1** Chemical structure of notoginsenoside (R1).

### Animals

C57/BL mice, weighing 22-26 g and aged 8-10 wk (the animal certificate number was SCXK 2002-2001) were purchased from the Animal Center of Peking University Health Science Center. The animals were caged at 24℃ ± 1℃ with a humidity of 50% ± 5% in a 12 h light/dark cycle, and starved with free access to water for 12 h before the experiment. All animals were handled according to the Guidelines of the Peking University Animal Research Committee.

### Intravital microscopy

C57/BL mice were anesthetized with 20% urethane (10 mL/kg body wt, im), as previously described[25]. The left jugular vein was cannulated for drug administration with a polyethylene pipe (0.96 mm in diameter). Immediately after laparotomy, the mice were placed on an observation board in lateral position. The liver was placed on an adjustable Plexiglas microscope stage within a thermo-controlled (37℃) observation box and carefully handled to minimize the influence of respiratory movements. The left lateral lobe of liver was observed under an inverted intravital microscope (DM-IRB, Leica, Germany) assisted by a 3CCD colour camera (JK-TU53H, 3CCD camera, Toshiba, Japan). Areas (400 μm × 320 μm) were selected that included both terminal portal venules

and central veins without adherent leukocytes for observation. Images of the microcirculation of liver surface were monitored through a × 20 objective, and the dynamics of hepatic microcirculation was recorded on DVD discs using a DVD recorder (DVR-R25, Malata, China). The liver surface for observation was moisturized with 37℃ physiological saline drops throughout the whole procedure, and the liver surface around the observation region was covered with saline-soaked cotton gauze[26].

### *Procedure for ischemia and reperfusion*

The surface of liver was observed for 10 min before ligation of the superior mesenteric artery (SMA) to ensure that all parameters measured were in a steady state. The SMA was then ligated with a snare created from polyethylene tubing (1.00 mm) for 15 min. After ischemia, the ligation was gently released for reperfusion. Venular diameter, RBC velocity, sinusoidal reperfusion, and leukocyte rolling and adhesion were determined immediately before ischemia (baseline) and every 15 min after reperfusion for half an hour.

### *Experimental protocols*

Mice in the I/R group were continuously infused with vehicle saline through the jugular vein from 10 min before I/R until the end of the observation. Mice in the R1 + I/R group were continuously infused with R1 (10 mg/kg per hour) from 10 min before I/R until the end of the observation. Mice in the sham-operated control group were treated in an identical fashion as those in the I/R group, but not subjected to ligation of the SMA. Six mice (three males, three females) were included in each group.

### *Determination of microcirculatory parameters*

The diameters of terminal portal venules and central veins were on replayed DVD images using Image-Pro Plus 5.0 software[27]. The result was presented as the ratio of the value determined at 15 min or 30 min to the baseline.

To access the sinusoidal perfusion, the number of hepatic sinusoids with red blood cells (RBCs) flowing through in the hepatic terminal portal venule and central vein regions was scored on the DVD replay, and presented as the perfused hepatic sinusoids/field of view (250 μm × 300 μm)[26]. The result was presented as the ratio of the value determined at 15 min or 30 min to the baseline.

The RBC velocity in hepatic terminal portal venule and central vein was recorded at a rate of 1000 frames/s by changing the monitor from CCD to a high speed video camera system (FASTCAM-ultima APX, photon, Japan), and the recordings were replayed from the high speed stored images at a rate of 25 frames/s. The RBC velocity in venules was measured with Image-Pro Plus 5.0 software[26,27]. The velocity was presented as μm/s. The result was presented as the ratio of the value determined at 15 min or 30 min to the baseline.

To evaluate the leukocyte rolling and adhesion the fluorescence tracer 0.2 mL Rhodamine 6G (0.5 mg/mL in physiological saline) was administrated *via* the left jugular vein for the selective staining of white blood cells *in vivo*[28-32]. Under the inverted laser confocal microscope system (BIO-RAD, Radiance 2100, A xiovert 200, Carl Zeiss Shanghai Co, Ltd, German), with 20 × fluorescent object lens, irradiated with the argon laser beam (wavelength = 543 nm), the rolling and adhesion of leukocytes in hepatic terminal portal venules and central veins were recorded. At each time point, a total of 10 successive frames were recorded at a scanning speed of 1 frame/s, and adhered leukocytes were defined as those that appeared at the same position in the 10 successive frames[24]. The adhered leukocytes in hepatic terminal portal venules and central veins were presented as the number of leukocytes/200 μm, while those within the hepatic sinusoid were counted as the number per field of view of 200 $\mu m^2$. The leukocytes that stayed at the same position in hepatic terminal portal venules and central veins for less than 10 s were designated as rolling leukocytes, and presented as the number/200 μm.

### *Analysis of immnofluorescent staining of hepatic endothelial adhesion molecules E-selectin and ICAM-1*

After 30-min reperfusion, liver was fixed with 4% paraformaldehyde perfusion, removed and frozen with liquid nitrogen, then cut into sections of 6 μm by a cryostat (LEICA CM 1800, Leica Co., German). The sections were further fixed with 4% paraformaldehyde at room temperature for 10 min and washed with PBS. The samples were then immunohistochemically stained as routing. Goat polyclonal antibody against mouse E-selectin or goat polyclonal antibody against mouse ICAM-1 was applied at dilution of 1:50. The secondary antibody (Rhodamine-labeled rabbit anti goat IgG diluted at 1:200) was added and incubated at 37℃ for 30 min, followed by washing with PBS and incubation with Hoechst 33342 (2 μg/mL) at room temperature for 3 min[33]. After washed with PBS, the specimen was sealed and observed under Laser confocal microscope with 63 × object lens. Fluorescence intensity was detected at excitation wavelength 543 nm for R-phycoerythrin and 405 nm for Hoechst (nuclear staining). Five fields of view ($1.6 \times 10^4$ $\mu m^2$ each) were evaluated in mouse hepatic sinusoids for each condition. Fluorescence intensities of E-selectin or ICAM-1 were estimated by Image Pro Plus software and expressed as an average proportion, positive area/area of one field of view ($1.6 \times 10^4$ $\mu m^2$).

### *Assessment of the expression of adhesion molecules CD11b and CD18 in peripheral neutrophils*

After 30-min reperfusion, blood was collected via inferior vena cava and anticoagulated with heparin (20 unit/mL whole blood). The sample was incubated with 1 μg FITC-labeled antibody against CD18 or CD11b for 20 min at room temperature in dark. RBCs were lysed by addition of hemolysin and the samples were then washed twice with PBS. Flow cytometry (FACS Calibur, B.D. Co, USA) was used to assess the mean fluorescence intensity of CD11b or CD18 for 5000 neutrophils in each condition[22].

### *Peripheral blood hepatic enzyme assay*

In some mice, at 30 min and 60 min, respectively after reperfusion, blood samples were withdrawn *via* inferior vena cava and anticoagulated with heparin (20 unit/mL whole blood). The blood serum was isolated by

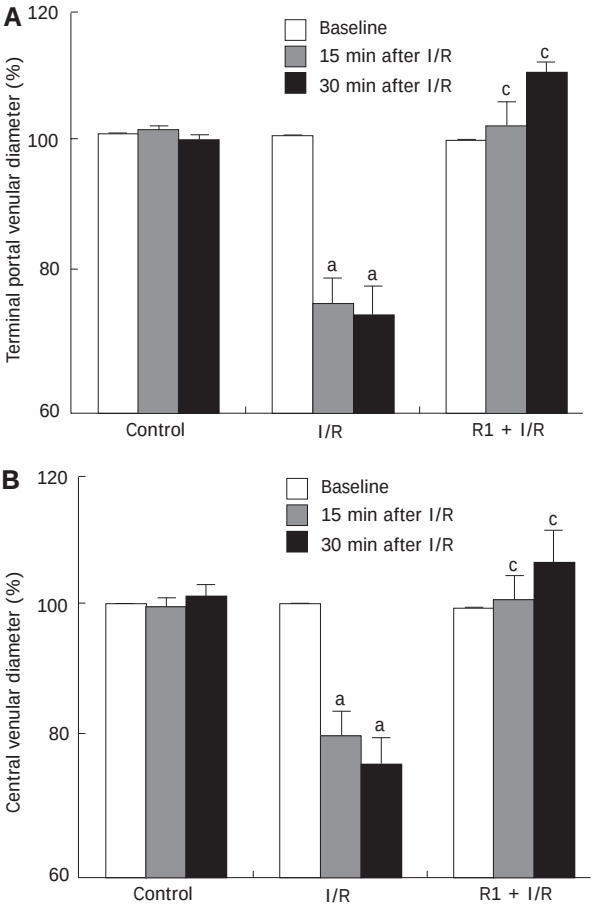


**Figure 2** Effect of R1 on the terminal portal venular diameter (**A**) and central venular diameter (**B**) of hepatic venules of mice subjected to SMA I/R at 0 min before ischemia (Baseline) and 15 min, 30 min after reperfusion. Abscissa represents the ratio of the diameter value at a time point to the baseline. The results are presented as mean ± SE from 6 animals. $^{a}P < 0.05$ *vs* control, $^{c}P < 0.05$ *vs* I/R.

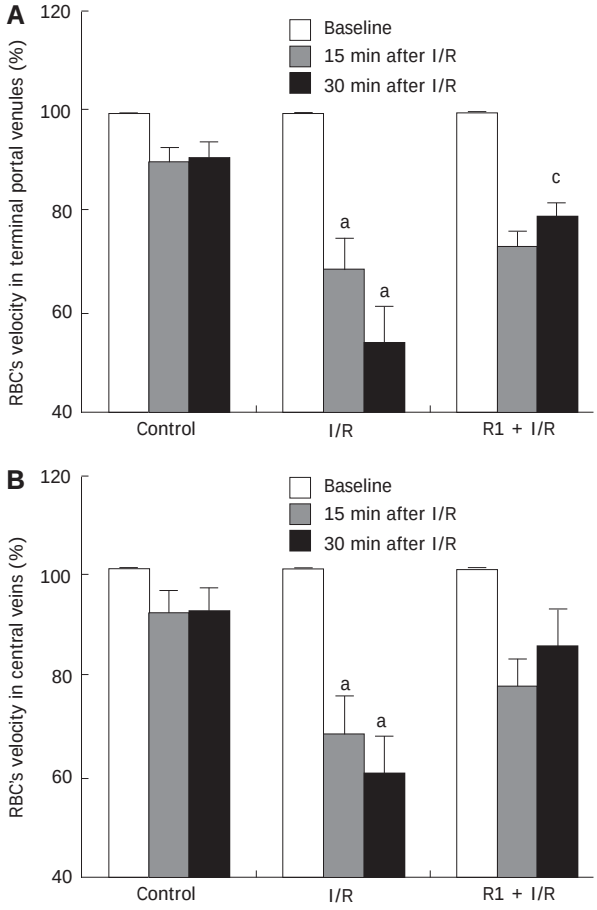


**Figure 3** Effect of R1 on the RBC velocity in terminal portal venlues (**A**) and in central veins (**B**) of mice subjected to SMA I/R at 0 min before ischemia (Baseline) and 15 min, 30 min after reperfusion. Abscissa represents the ratio of the RBC velocity value at a time point to the baseline. The results are presented as mean ± SE from 6 animals. $^{a}P < 0.05$ *vs* control, $^{c}P < 0.05$ *vs* I/R.

centrifugation (Allegra™ 64R Centrifuge, Beckman Coultertm, German) at 4000 r/min for 10 min at 4℃ and stored at -20℃. The activities of LDH, ALT, and AST were measured respectively using lactate dehydrogenase, alanine aminotransferase and aspartate aminotransferase kits with parameter rate-A[34], following their manufacturer's instructions, with an automatic enzyme analyzer (7170A Automatic Analyzer, Hitachi, Japan).

### *Peripheral blood TNF-α, IL-6 and MCP-1 assay*

At 30 min after reperfusion, blood was collected *via* inferior vena cava, and anticoagulated with heparin (20 unit/mL whole blood). The blood serum was isolated by centrifugation (Allegra™ 64R Centrifuge, Beckman Coultertm, German) at 4000 r/min for 10 min at 4℃ and stored at -20℃. The concentrations of TNF-α, IL-6 and MCP-1 were measured by flow cytometry with a BD cytometric bead array kit (BD Biosciences Pharmingen, USA)[35]. Fifty μL bead was added into 50 μL blood plasma or standard substance and incubated at room temperature in dark for 1 h for bead capture. Fifty μL PE-labelled detecting antibody was then added and incubated at room temperature for 2 h to form a sandwich complex. After incubation, the samples were washed thoroughly with 1 mL washing buffer (BD Biosciences Pharmingen, USA). The mean fluorescence intensity of TNF-α, IL-6 and MCP-1 was detected respectively by flow cytometry (FACS Calibur, B.D. Co., USA) and the data were analyzed using the BD cytometric bead array analysis software.

### *Statistical analysis*

Values are presented as mean ± SE ($n = 6$), *F*-test was performed using SPSS 10.0 statistical software. $P < 0.05$ was considered statistically significant.

## RESULTS

The effect of R1 on the diameter of hepatic terminal portal venule and central vein of mice subjected to SMA I/R are shown in Figure 2. In the control group, the diameters of both terminal portal venules and central veins remained nearly constant over the entire observation period. SMA I/R decreased the diameter of vessels in a time-dependent manner, while treatment with R1 significantly relieved SMA I/R-induced decrease in the vessel diameters.

The influence of R1 on the RBC velocity in hepatic terminal portal venules and central veins of mice after SMA I/R is shown in Figure 3. In the control group, no significant change was observed in the RBC velocity of both

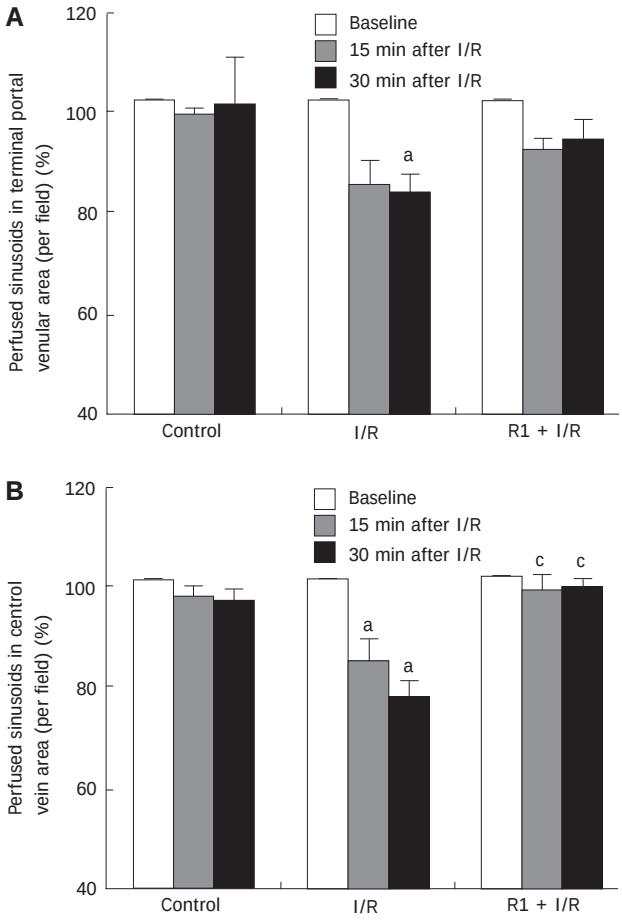


**Figure 4** Effect of R1 on the perfused hepatic sinusoids in the areas of terminal portal venules (**A**) and in the area of central veins (**B**) of mice subjected to SMA I/R at 0 min before ischemia (Baseline) and 15 min, 30 min after reperfusion. Abscissa represents the ratio of perfused sinusoids at a time point to the baseline. The results are presented as mean ± SE from 6 animals. $^{a}P < 0.05$ *vs* control, $^{c}P < 0.05$ *vs* I/R.

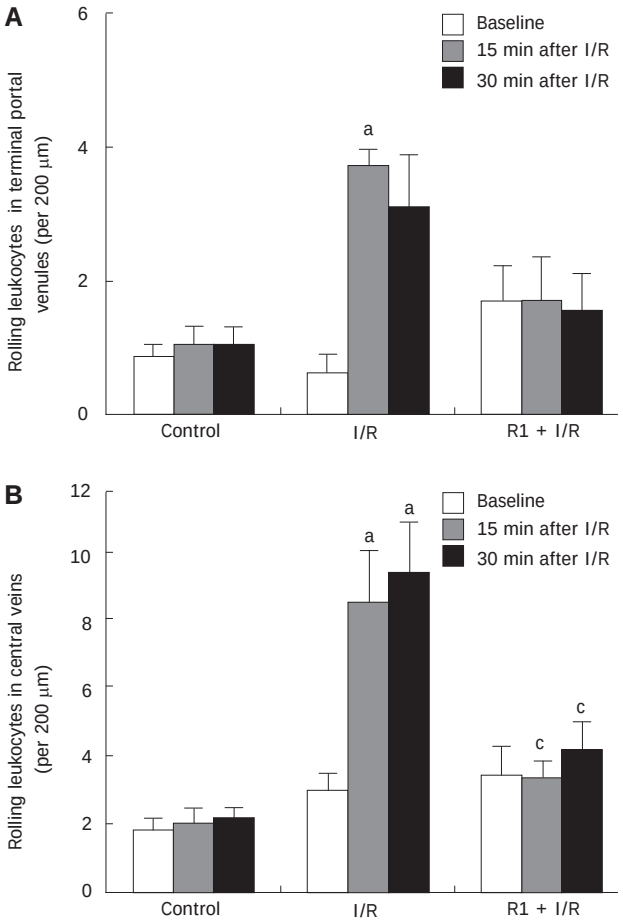


**Figure 5** Effect of R1 on the rolling leukocytes in the areas of terminal portal venlues (**A**) and central veins (**B**) of mice subjected to SMA I/R at 0 min before ischemia (Baseline) and 15 min, 30 min after reperfusion. Abscissa represents the number of rolling leukocytes per 200 μm. The results are presented as mean ± SE from 6 animals. $^{a}P < 0.05$ *vs* control, $^{c}P < 0.05$ *vs* I/R.

types of vessels during the period of observation. SMA I/R significantly decreased the RBC velocity of vessels in a time-dependent fashion. R1 treatment blunted the SMA I/R-induced decrease in the RBC velocity of both types of vessels at 30 min after reperfusion, being significant in terminal portal venules (Figure 3A) but not in central veins (Figure 3B), in comparison with the I/R group.

The effect of R1 on the number of reperfused sinusoids in hepatic terminal portal venule and central vein areas after SMA I/R is depicted in Figure 4. In the control group, no significant change in the number of reperfused sinusoids was detected either in the terminal portal venule area or in the central vein area through the entire observation. SMA I/R exposure elicited a time-dependent decrease in the number of reperfused sinusoids, which became statistically significant at 15 min of reperfusion in the central vein areas and at 30 min of reperfusion in the terminal portal venule areas compared to the control group. I/R-induced decrease in the number of reperfused sinusoids in central vein areas was attenuated significantly after treatment with R1 (Figure 4B), and this effect was not observed in the terminal portal venule areas (Figure 4A).

The effect of R1 on the number of rolling leukocytes in mouse hepatic terminal portal venules and central veins after SMA I/R is depicted in Figure 5. I/R challenge significantly increased the number of rolling leukocytes in both terminal portal venules and central veins when compared with the control group, although a small number of rolling leukocytes could be observed in the control group during the period of examination. R1 treatment reduced the enhancement in the leukocyte rolling induced by I/R, which was statistically significant in the central veins (Figure 5B), but not in terminal portal venules (Figure 5A).

Figure 6 shows the effect of R1 on the adhesion of leukocytes induced by SMA I/R. SMA I/R increased the number of adherent leukocytes in both hepatic terminal portal venule and central vein regions, which was attenuated after treatment with R1, being significant in the terminal portal venules (Figure 6A), but not in the central veins (Figure 6B).

The effect of R1 on the leukocyte adhesion in sinusoids of hepatic terminal portal venule and central vein areas was determined (Figure 7). As in the hepatic terminal portal venules and central veins, only a small number of adherent leukocytes were visualised within sinusoids either of either the terminal portal venule area or the central vein area in the control group. A remarkable increase in the number of adherent leukocytes within sinusoids of

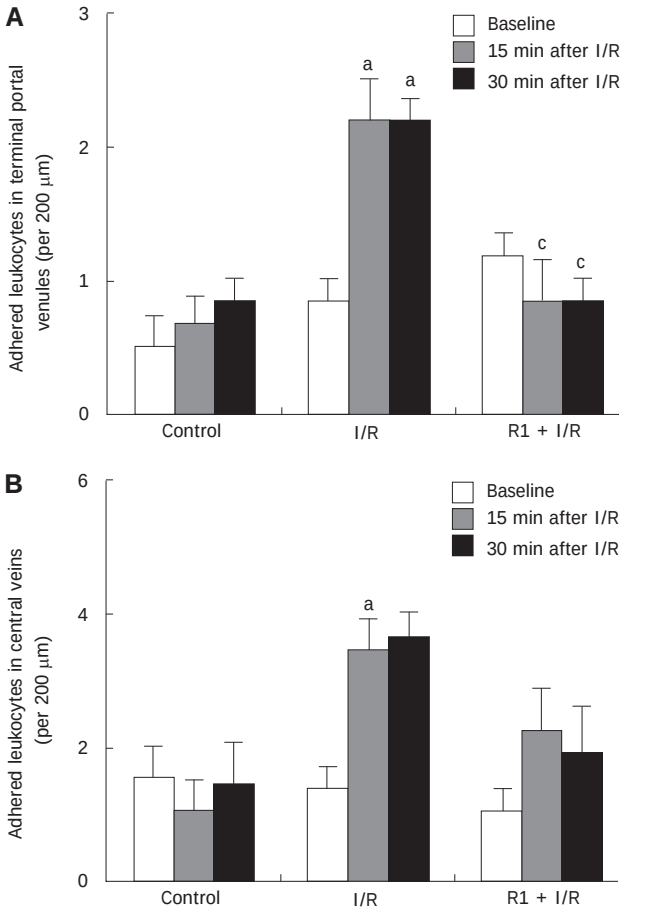


**Figure 6** Effect of R1 on the adherent leukocytes in terminal portal venlues (**A**) and central veins (**B**) of mice subjected to SMA I/R at 0 min before ischemia (Baseline) and 15 min, 30 min after reperfusion. Abscissa represents the number of adherent leukocytes per 200 μm. The results are presented as mean ± SE from 6 animals. $^{a}P < 0.05$ *vs* control, $^{c}P < 0.05$ *vs* I/R.

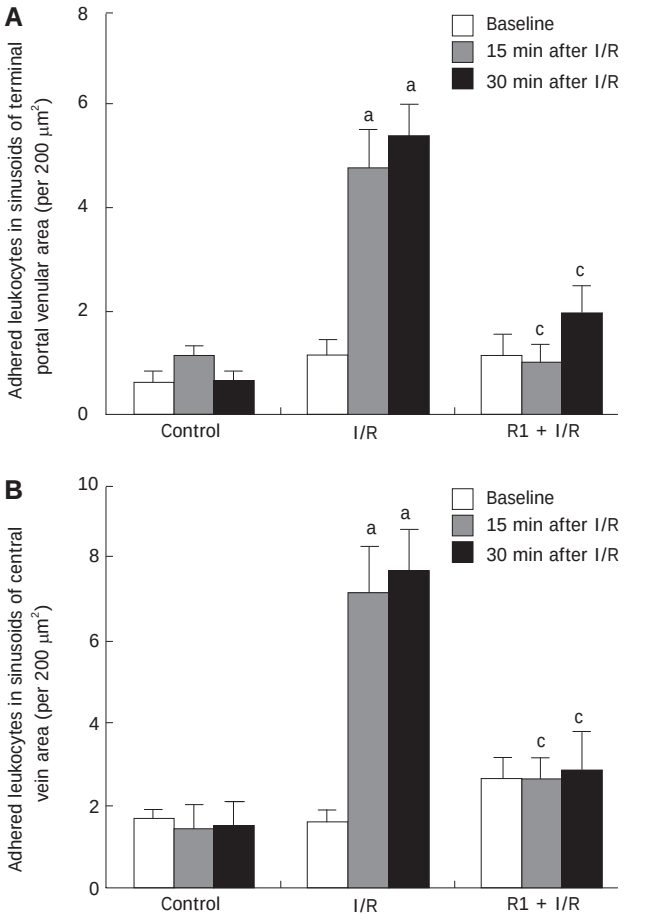


**Figure 7** Effect of R1 on the adherent leukocytes in the hepatic sinusoids in the areas of terminal portal veins (**A**) and central veins (**B**) of mice subjected to SMA I/R at 0 min before ischemia (Baseline) and 15 min, 30 min after reperfusion. Abscissa represents the number of adherent leukocytes per field of view of 200 μm². The results are presented as mean ± SE from 6 animals. $^{a}P < 0.05$ *vs* control, $^{c}P < 0.05$ *vs* I/R.

both areas was observed when the mice were subjected to SMA I/R, which was inhibited significantly after treatment with R1 (Figure 7) starting from 15 min after reperfusion.

Figure 8 shows the expression of E-selectin in mouse hepatic sinusoids in the sham, I/R and R1 + I/R groups after 30 min of reperfusion. Thirty minutes after reperfusion, the expression of E-selectin increased (Figure 8B) compared with the sham group (Figure 8A), and treatment with R1 (Figure 8C) suppressed the increase in I/R-elicited E-selectin expression.

The influence of R1 on the expression of E-selectin in mouse hepatic sinusoids after 30 min of reperfusion was quantitatively evaluated (Figure 9A). Thirty-minute reperfusion significantly enhanced the expression of E-selectin. The SMA I/R-induced increase in the expression of E-selectin was completely ablated after treatment with R1.

The expression of ICAM-1 in mouse hepatic sinusoids after 30 min of reperfusion had no significant change. Treatment with R1 had no significant influence on the expression of ICAM-1 either (data not shown).

After 30 min of reperfusion, the blood was collected and used to evaluate the role of R1 in the expression of adhesion molecules CD18 and CD11b of mouse peripheral neutrophils. As illustrated in Figure 9B, 30-minute reperfusion significantly enhanced the expression of both CD18 and CD11b compared to the control group. R1 treatment significantly inhibited the increment in the mean fluorescence intensity of CD18 induced by I/R, and also diminished, although not significantly, the increment in the fluorescence intensity of CD11b induced by I/R.

The influence of R1 on the concentration of enzymes in mouse peripheral blood was determined after 30 and 60 min of reperfusion (Figure 10). Thirty-minute or 60-minute reperfusion obviously increased the concentration of LDH, ALT and AST. However, treatment with R1 could not significantly blunt these increases, except for the activity of AST after 60-min reperfusion. I/R significantly increased the concentrations of IL-6 and MCP-1, but not TNF-α compared to the control group. R1 did not influence the increase in the concentration of IL-6 and MCP-1 induced by I/R (data not shown).

## DISCUSSION

Terminal portal venules and central veins are two major types of vessels consisting of hepatic microvasculature, in addition to sinusoids. Using intravital microscopy, the present study demonstrated that the mouse SMA I/R induced a variety of disorders in hepatic microcirculation,

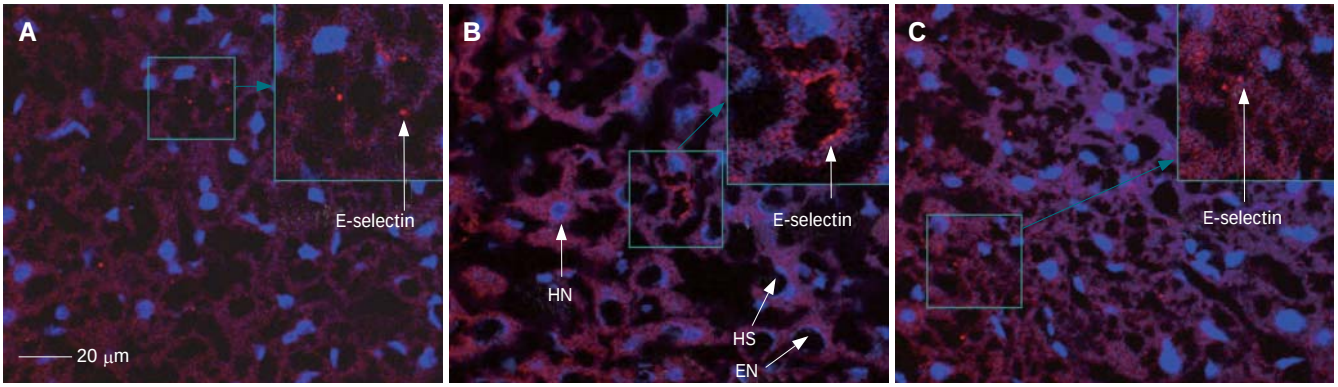


**Figure 8** Expression of E-selectin in mouse hepatic sinusoid in sham (**A**), I/R (**B**) and R1 + I/R (**C**) after 30 min of reperfusion. HN: hepatocyte nucleus; EN: endothelium nucleus; HS: hepatic sinusoid; Bar indicates 20 μm.

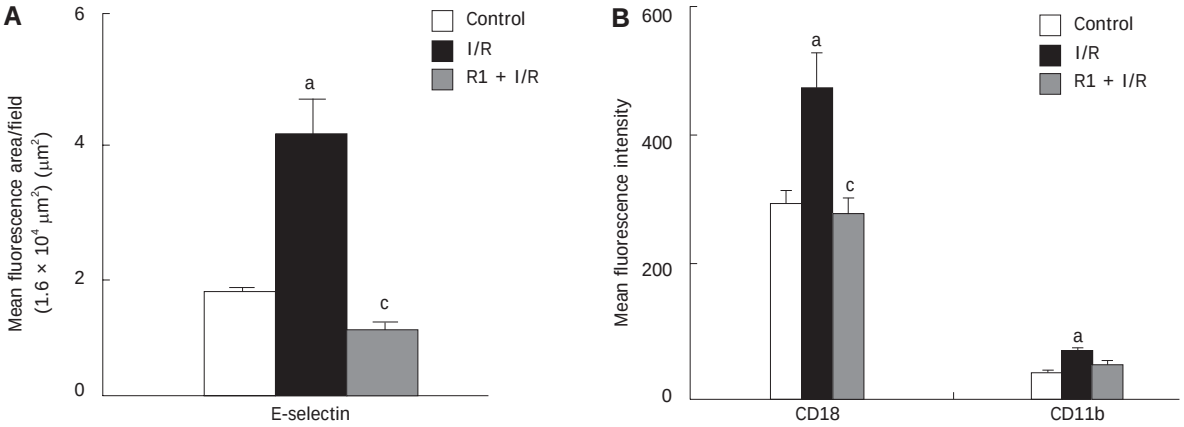


**Figure 9** Effect of R1 on the expression of E-selectin in hepatic vessels (**A**) and CD18 and CD11b in neutrophils (**B**) of mice subjected to SMA I/R. The results are presented as mean ± SE from 6 animals. $^{a}P < 0.05$ *vs* control, $^{c}P < 0.05$ *vs* I/R.

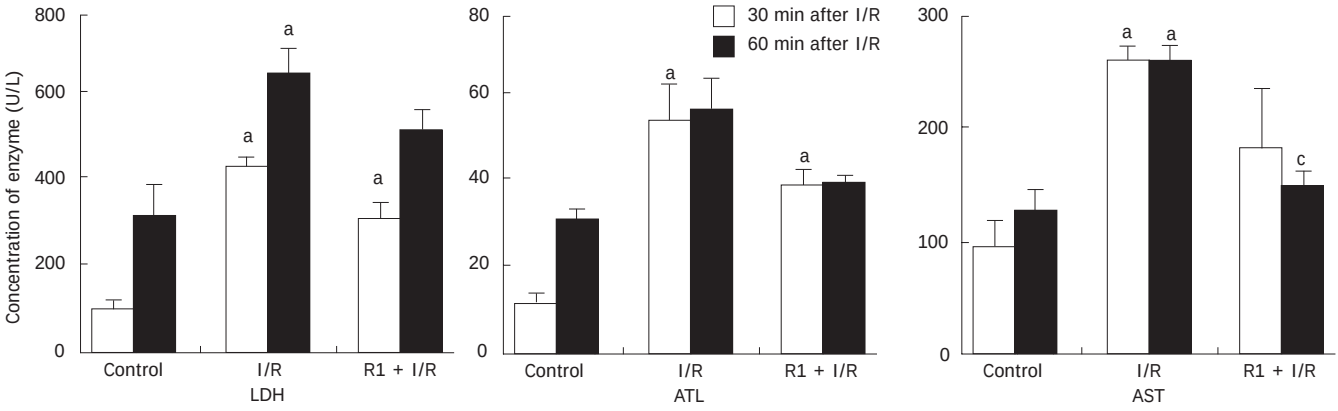


**Figure 10** Effect of R1 on the concentration of LDH, ALT and AST in serum of mice subjected to SMA I/R. The results are presented as mean ± SE from 6 animals. $^{a}P < 0.05$ *vs* control, $^{c}P < 0.05$ *vs* I/R.

including the decreased diameters of terminal portal venules and central veins, RBC velocity in venules and the number of perfused sinusoids. Besides, leukocyte rolling and adhesion in hepatic venules and sinusoids were also promoted by the SMA I/R challenge. These results are in agreement with previous findings[2,4,24-26,30]. In the current study, treatment with R1 could remarkably attenuate hepatic microcirculatory disturbances in mice evoked by SMA I/R. PNS improved gut I/R-induced hepatic microcirculation disturbances and CP inhibited the adhesion of leukocytes to the hepatic vascular endothelium in rats induced by gut I/R, suggesting that R1 is at least one of the components of PNS that are responsible for its beneficial effect on hepatic microcirculation[19,24].

The concentration of ET-1 increases in serum and hepatic parenchyma in response to I/R[36], while NO is depleted by combination with I/R-evoked $^{-}O^{\cdot-}$[37], which concurs to bring about unbalance between ET-1

and NO, resulting in contraction of hepatic vessels. The present study revealed that administration of R1 attenuated I/R-elicited contraction of hepatic terminal portal venules and central veins that profoundly improved microcirculation in mice, suggesting that R1 may exert its action on the production of ET-1 and NO in the situation of I/R, which merits further study.

I/R-induced microcirculatory dysfunction and subsequent tissue injury are a complicated process consisting of multiple reactions, among which leukocyte recruitment is a crucial step mediated by the expression of a group of adhesion molecules on neutrophils and endothelial cells. It was reported that peroxide produced by SMA I/R degrades I-κB for activation of NF-κB and induces the expression of vascular endothelial E-selectin and ICAM-1[6-10], and also transfers L-selectin and adhesion molecules CD11b and CD18 from leukocyte cytoplasm to the cell surface[11-13,38], which initiates leukocyte rolling and adhesion ultimately. The ability of R1 to attenuate gut I/R-induced leukocyte rolling and adhesion in hepatic venules found in the present study is most probably due to its inhibiting effect on the expression of adhesion molecules on both leukocytes and endothelial cells, as suggested by the fact that pre-treatment with R1 could significantly blunt SMA I/R-induced expression of E-selectin on endothelium and CD18 on neutrophils.

It has been demonstrated that SMA I/R provokes a surge of peroxide release which activates NF-κB[6,7], leading not only to the expression of adhesion molecules on leukocytes and endothelium and subsequent leukocyte rolling and adhesion, but also to the explosive release of cytokines from leukocytes, endothelial and Kupffer cells[39-41], including TNF-α, IL-6 and MCP-1. The present experiment revealed that mouse gut I/R significantly increased the concentration of IL-6 and MCP-1, but not TNF-α in plasma. The reason is so far unknown. Moreover, no study is available to date concerning the antioxidant effect of R1 in I/R-induced hepatic microcirculatory disturbance, and it merits clarification if taking into account the fact that R1 depresses I/R-induced expression of adhesion molecules, but does not interfere with the increased concentrations of IL-6 and MCP-1 induced by I/R.

As expected, results of the present study show that the activities of ALT, AST and LDH of peripheral blood could slightly increased in response to 60-min SMA I/R, indicating that intestine I/R-induced microcirculatory disturbance in liver leads to injury of hepatic cells and dysfunction of the liver. Treatment with R1 significantly suppressed the activity of AST 60 min after reperfusion, suggesting that R1 can protect against hepatocyte injury induced by I/R. This beneficial effect is most likely related to its improving effect on hepatic microcirculatory disturbance, as mentioned above.

In summary, treatment with R1 considerably attenuates SMA I/R-induced hepatic microcirculatory disturbances, including decreased venular diameters, RBC velocity, the number of perfused sinusoids, as well as the increased leukocyte rolling and adhesion. R1 ameliorates SMA I/R-induced increase in the leukocyte rolling and adhesion in hepatic venules by inhibiting the expression of adhesion molecules on endothelial cells and neutrophils. We propose that it is the improving effect of R1 on microcirculatory disturbance that underlines its protecting function against I/R-induced hepatic injury.

## COMMENTS

### Background

Major abdomen surgery or organ transplantation initiates gut ischemia and reperfusion (I/R) leading to hepatic microcirculatory disturbance and subsequent liver injury, a manifestation that is closely correlated to the outcome of operation or transplantation and the living quality of patients as well. Thus, attenuating hepatic microcirculatory disturbance and liver injury elicited by gut I/R is of pivotal significance in clinic. Notoginsenoside R1 (R1), one of the saponins derived from *Panax notoginseng*, is reported to attenuate endotoxin-induced mesenteric microcirculatory disturbance in rats by inhibiting oxygen peroxide production and expression of adhesion molecules CD11b/CD18. It has been shown that R1 containing compound Chinese medicine preparation (cardiotonic pills) is able to ameliorate hepatic microcirculatory disturbance and liver injury elicited by gut I/R. R1 containing Chinese medicines is extensively applied in treatment of microcirculatory disturbance related diseases in China. However, no report is available regarding its attenuating effect on hepatic microcirculatory disturbance and liver injury induced by gut I/R.

### Research frontiers

In present study, an animal model of hepatic microcirculatory disturbance was established by ligation of the superior mesenteric artery (SMA) in C57/BL mice for 15 min followed by 30-min reperfusion. The dynamics of vascular diameter, RBC velocity, rolling and adherent leukocytes was investigated in the hepatic terminal portal venule and central vein regions under inverted intravital microscope assisted by a 3CCD color camera and high speed video camera as well as a laser confocal microscope. Thirty minutes after reperfusion, the expression of adhesion molecules CD11b and CD18 on leukocytes in peripheral blood was estimated, and the expression of E-selectin and ICAM-1 in hepatic tissue was determined by immunohistochemistry. Sixty minutes after reperfusion, the levels of LDH, ALT, AST in peripheral blood were measured to explore the possible protective effect of R1 on hepatic microcirculatory disturbance and liver injury elicited by gut I/R.

### Innovations and breakthroughs

By using a visualized microcirculatory research, results of the present study provide evidence for the first time that a pulse prior administration of R1 is able to prevent hepatic microcirculatory disturbance in mice induced by intestine I/R, including ameliorating contraction of hepatic terminal portal venule and central vein, resuming reduced velocity of RBCs, and inhibiting leukocyte rolling and adhesion in hepatic terminal portal venule and central vein, the later may be correlated to the inhibition of the expression of adhesion molecules and E-selectin on vascular endothelial cells and CD18 on leukocytes. R1 treatment could suppress AST level in peripheral blood, suggesting that R1 is able to prevent intestine I/R-induced hepatic microcirculatory disturbance and subsequent liver injury.

### Applications

The findings in the present work support the utilization of R1 as a remedy in clinic to prevent hepatic microcirculatory disturbance and liver injury following major abdomen surgery or transplantation.

### Peer review

This is a very interesting paper examining the effects of notoginsenoside R1 (R1) on hepatic microvascular function after gut I/R. The data are largely convincing. R1 has both vasodilatory and anti-inflammatory functions.

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*CLINICAL RESEARCH*

# Increased susceptibility for intrahepatic cholestasis of pregnancy and contraceptive-induced cholestasis in carriers of the 1331T>C polymorphism in the bile salt export pump

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## Abstract

**AIM:** To study the association of three common *ABCB11* and *ABCC2* polymorphisms (*ABCB11*: 1331T>C → V444A; *ABCC2*: 3563T>A → V1188E and 4544G>A → C1515Y) with intrahepatic cholestasis of pregnancy (ICP) and contraceptive-induced cholestasis (CIC).

**METHODS:** *ABCB11* and *ABCC2* genotyping data were available from four CIC patients and from 42 and 33 ICP patients, respectively. Allele-frequencies of the studied polymorphisms were compared with those in healthy pregnant controls and Caucasian individuals. Furthermore, serum bile acid levels were correlated with the presence or absence of the 1331 C allele.

**RESULTS:** The *ABCB11* 1331T>C polymorphism was significantly more frequent in cholestatic patients than in pregnant controls: C allele 76.2% (CI, 58.0-94.4) *vs* 51.3% (CI 35.8-66.7), respectively ($P$ = 0.0007); and CC allele 57.1% (CI 36.0-78.3) *vs* 20% (CI 7.6-32.4), respectively ($P$ = 0.0065). All four CIC patients were homozygous carriers of the C allele. In contrast, none of the studied *ABCC2* polymorphism was overrepresented in ICP or CIC patients. Higher serum bile acid levels were found in carriers of the 1331CC genotype compared to carriers of the TT genotype.

**CONCLUSION:** Our data support a role for the *ABCB11* 1331T>C polymorphism as a susceptibility factor for the development of estrogen-induced cholestasis, whereas no such association was found for *ABCC2*. Serum bile acid and γ-glutamyl transferase levels might help to distinguish *ABCB4*- and *ABCB11*-related forms of ICP and CIC.



**Key words:** Cholestasis of pregnancy; Contraceptive-induced cholestasis; Bile salt export pump; Multidrug resistance associated protein 2; Pharmacogenetics

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Meier Y, Zodan T, Lang C, Zimmermann R, Kullak-Ublick GA, Meier PJ, Stieger B, Pauli-Magnus C. Increased susceptibility for intrahepatic cholestasis of pregnancy and contraceptive-induced cholestasis in carriers of the 1331T>C polymorphism in the bile salt export pump. *World J Gastroenterol* 2008; 14(1): 38-45

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## INTRODUCTION

Intrahepatic cholestasis of pregnancy (ICP) and oral contraceptive-induced cholestasis (CIC) are two acquired forms of cholestasis, which are observed in otherwise healthy young women with a normal medical history. Both syndromes are rapidly reversible upon discontinuation of the hormonal challenge, which suggests that female sex hormones play a key pathogenic role in these forms of cholestasis[1,2]. In line with these observations, ICP usually occurs during the third trimester of pregnancy, when serum concentrations of estrogens and progesterone reach their peak[3,4]. Furthermore, women with ICP and female family members of ICP patients have an increased susceptibility to develop intrahepatic cholestasis under oral contraception[5].

A genetic predisposition for both types of hormonal cholestasis has been suspected based upon the strong regional clustering[6], the higher prevalence in female family members of patients with ICP[5,7] and the co-incidence with hereditary cases of progressive familial intrahepatic cholestasis[5,7]. Recently, mutations in the *ABCB4* gene that encodes the canalicular phospholipid flippase multidrug resistance protein

3 (MDR3) have been implicated in the development of ICP and CIC in a subset of affected patients[5,8-12]. MDR3-associated cases of hormonal cholestasis are associated with elevated serum γ-glutamyl transferase (γ-GT) levels in 80% of affected patients, which reflects cholangiocytic damage characteristic of MDR3 dysfunction[11].

In the same study, the majority of ICP women without *ABCB4* mutations exhibited normal γ-GT levels[11], which suggests a different pathogenic mechanism in this subset of patients. Dysfunction of the bile salt export pump (BSEP) or the multidrug resistance associated protein 2 (MRP2) have, therefore, been proposed as alternative candidate proteins involved in the pathogenesis of hormonal cholestasis. BSEP constitutes the predominant bile salt efflux system of hepatocytes, which mediates the cellular excretion of numerous conjugated bile salts into the bile canaliculus[13-16]. In contrast, the bilirubin transporter MRP2 is the main driving force for bile-salt-independent bile flow through canalicular excretion of reduced glutathione[17,18]. Given their important roles in bile formation and bilirubin secretion, inherited and acquired dysfunction of these proteins can lead to severe cholestatic syndromes and conjugated hyperbilirubinemia, respectively[19-21].

In hormonal cholestasis, *in vitro* inhibition of BSEP by estrogen and progesterone metabolites has been proposed as an underlying pathophysiological mechanism[22]. BSEP inhibition by estrogen and progesterone metabolites takes place from the luminal side of the bile canaliculus (so-called trans inhibition), which requires previous MRP2-mediated canalicular secretion of conjugated metabolites[22,23]. Therefore, MRP2 dysfunction might contribute to this form of cholestasis. While sequencing of *ABCB11* in unrelated ICP women has not revealed the presence of disease-causing BSEP mutations[11], only little attention has so far been paid to the possible pathogenic role of functional *ABCB11* and *ABCC2* polymorphisms. Recent observations have suggested that a non-synonymous polymorphism in exon 13 of the *ABCB11* gene (1331T>C) is overrepresented in drug-induced cholestatic liver injury[24]. The same polymorphism has recently been observed more frequently in ICP women compared to healthy controls, pointing towards a possible role of this polymorphism as a susceptibility factor for ICP and CIC[25]. Furthermore, two non-synonymous *ABCC2* polymorphisms (V1188E and C1515Y) showed significant differences in hepatic MRP2 expression levels compared to the wildtype sequence, which could be relevant for the extent of BSEP trans inhibition[25].

The aim of the present study was, therefore, threefold: (1) to compare allele frequencies of the aforementioned *ABCB11* and *ABCC2* polymorphisms in a prospectively recruited group of patients with ICP and CIC; (2) to define the relative risk of the different polymorphisms for the development of ICP; and (3) to determine the extent of the increase in serum bile acid levels as marker of cholestasis in the presence of the different *ABCB11* 1331T>C genotypes.

## MATERIALS AND METHODS

### *Patients and controls*

After approval by the Ethics Committee of the University Hospital of Zurich and written informed consent from all participating individuals, blood samples for DNA extraction were obtained from Caucasian patients with ICP or CIC. The total population of analyzed individuals consisted of two different groups: 25 patients (21 $ICP_{new}$ patients and four CIC patients) were prospectively recruited for this study, and a second group of 20 patients ($ICP_{old}$) had already been described in a previous study by Pauli-Magnus and coworkers[11].

Two hundred and five Caucasian volunteers and patients without cholestasis, as well as Caucasian women with uneventful pregnancies ($n$ = 40), served as a control population for BSEP (*ABCB11*) and MDR3 (*ABCB4*) genetic variants. These controls have already been described in previous studies[11,25,26]. Specifically, pregnant controls were all healthy, as defined by normal serum levels of transaminases, bilirubin, γ-GT, alkaline phosphatase (AP) and bile acids. Caucasian controls from the study of Pauli-Magnus and coworkers[26] ($n$ = 95) were healthy volunteers recruited for participation in phase I studies, with uneventful medical history and normal blood biochemistry. Neither of these two control groups took any regular medication. In the case of the Caucasian control population of Meier and coworkers[25] ($n$ = 110), most patients suffered from extrahepatic malignancies, and cholestatic disease was excluded in all patients. Furthermore, none of these patients used medication known to be associated with the development of cholestasis.

For lack of DNA availability, only 110 out of 205 Caucasian controls could be used for MRP2 sequencing. For the same reason, a new group of Caucasian women with uneventful pregnancies ($n$ = 42) had to be collected for the MRP2 variants. Demographic data and pregnancy course of these women did not differ from the previous control group.

Diagnosis of ICP was based upon: (1) a clinical history of pruritus, which occurred in the third trimester of pregnancy; (2) the presence of laboratory abnormalities suggestive of ICP: fasting serum bile acid $\geqslant$ 1.5 ULN (upper limit of normal) and/or serum AP levels $\geqslant$ 1.5 ULN and/or alanine aminotransferase (ALT) levels $\geqslant$ 1.5 ULN; and (3) spontaneous resolution of clinical symptoms and laboratory findings after delivery. Diagnosis of CIC was based upon laboratory abnormalities as defined for ICP and the exclusion of preexisting liver disease defined by: (1) a negative serology for hepatitis A, B and C; (2) the exclusion of other preexisting medical conditions that could explain liver injury, such as congestive heart failure, systemic infection, or malignancy; (3) normal liver ultrasound; and (4) a clear causal relationship to drug intake. Each case of ICP and CIC was evaluated by at least one obstetrician and one hepatologist, as well as by a clinical pharmacologist.

Full length *ABCB4* and *ABCB11* sequencing data were already available from the control groups, as well as from $ICP_{old}$ patients. To allow detection of additional *ABCB4* and *ABCB11* mutations in the new group, complete sequencing of these two genes was also performed in the 25 newly recruited patients. Genotyping of *ABCC2* included all CIC patients, as well as 17 out of 21 patients from the $ICP_{new}$ group and 16 of 21 patients in the $ICP_{old}$ group, which yielded a total number of 33 patients for *ABCC2*

**Table 1 Primers and probes of real-time PCR for allelic discrimination of *ABCC2* SNPs in Caucasians**

| cDNA position[1] | SNP | Exon | Amino acid change | Eense-/antisense primer | Probes[2] |
|---|---|---|---|---|---|
| 1249 | G>A | 10 | V417I | 5'-CCAACTTGGCCAGGAAGGA-3'/ | VIC 5'-CTGTTTCTCCAACGGTGTA-3' |
| | | | | 5'-GGCATCCACAGACATCAGGTT-3' | FAM 5'-ACTGTTTCTCCAATGGTGTA-3' |
| 3563 | T>A | 25 | V1188E | 5'-GCACCAGCAGCGATTTCTG-3'/ | VIC 5'-ACACAATGAGGTGAGGAT-3' |
| | | | | 5'-AGGTGATCCAGGAAAAGACACATTT-3' | FAM 5'-ACAATGAGGAGAGGAT-3' |
| 4544 | G>A | 32 | C1515Y | 5'-GTAATGGTCCTAGACAACGGGAAG-3'/ | VIC 5'-AGAGTGCGGCAGCC-3' |
| | | | | 5'-CCAGGGATTTGTAGCAGTTCTTCAG-3' | FAM 5'-ATTATAGAGTACGGCAGCC-3' |

[1]cDNA sequence from GenBank accession numbers NM_000392 starting at the ATG; [2]For each SNP two probes were designed and labeled with the fluorescent reporter dyes VIC (allele 1) and FAM (allele 2). SNP: Single nucleotide polymorphism.

genotyping. In nine patients (four $ICP_{new}$ and five $ICP_{old}$), no *ABCC2* genotyping could be performed for lack of DNA availability.

### Sequencing and genotyping

Isolation of DNA and DNA sequencing was done at Epidauros Biotechnology AG, Bernried, Germany. Genomic and cDNA sequences were derived from known sequences (*ABCB4*: AC005068.2 for non-coding exons -3 to 1 and coding exons 2 and 3; AC006154.1 for exons 4 to 12; AC0005045.2 for exons 13 to 28; and NM_000443.2 for cDNA; *ABCB11*: GenBank accession number AC008177.3 for promoter and exons 1 to 21; AC069165.2 for exons 22 to 28 and NM_003742.2 for cDNA).

**ABCB4 and ABCB11:** Sequencing of *ABCB4* covered a proximately 8000 bp, including (1) 2000 bp of the upstream promoter region and non-coding exon -3 to 1 and, (2) coding exons 2-28 and 100-350 bp of the intronic sequence around each exon. For *ABCB11*, sequencing covered 10000 bp including (1) non-coding exon 1 and 2400 bp of the upstream promoter region and, (2) coding exons 2-28 and 100-350 bp of the intronic sequence around each exon. Primers for genomic DNA were designed to span all exons and at least 100 bp of the flanking intronic sequence at the 5' and 3' end of each exon. The DNA sequence of purified PCR fragments was analyzed on an ABI3700 capillary sequencer (ABI, Weiterstadt, Germany) and assembled using the phredPhrap, Consed and PolyPhred software (University of Washington). Details regarding the primers, optimized PCR conditions and subsequent purification and sequencing of the fragments are available at info@epidauros.com.

**ABCC2:** Three non-synonymous polymorphisms with a potential impact on MRP2 function and expression were chosen for genotyping[25]: 1249G>A variant (V417I, rs2273697), 3563T>A (V1188E, rs17222723) and 4544G>A (C1515Y, rs8187710). Genotyping was performed with the Custom TaqMan SNP Genotyping Assays procedure (Applied Biosystems, Foster City, CA, USA) which contained a sense- and an antisense primer and two probes, labeled with fluorescent reporter dyes, either VIC or 6-Fam at the 5' end and a non-fluorescent quencher at the 3' end to distinguish between alleles 1 and 2, respectively. Primer and probe sequences for individual SNPs are given in Table 1. Probe solution (0.625 μL) and 12.5 μL of 2 × Universal PCR Master Mix (Applied Biosystems) were brought to 25 μL with 20 ng of genomic DNA. PCR reaction (2 min at 50℃, followed by 10 min at 95℃ and 40 cycles of 15 s at 92℃ and 1 min at 60℃). Allelic discrimination was processed with an ABI PRISM 7700 Sequence Detector.

### Statistical analysis

Genotype distribution, allelic frequencies and odds ratios (ORs) are given with 95% CI. In *ABCB11*, formal statistical analysis was only performed for the 1331T>C polymorphisms (rs2287622), whereas for *ABCC2* analysis, it included two highly linked polymorphisms. No correction according to Bonferroni was, therefore, required. Differences investigated in our study apply to a proportion of diseased *versus* non-diseased individuals within the whole population, using an unmatched case control design. Response (ICP *versus* non-ICP) and predictors (T *versus* C) were both binary variables and were therefore best condensed into a 2 × 2 table. Differences in genotype distribution between patients and controls were calculated with the $\chi^2$ test, and difference in allelic frequencies between two groups was performed using a 2 × 2 Fisher exact test. $P \leqslant 0.05$ was considered statistically significant.

## RESULTS

### Patient characteristics

A total of 25 unrelated patients with estrogen-associated intrahepatic cholestasis were prospectively enrolled in this study, 21 with ICP and four with oral CIC. Demographic data and laboratory findings in ICP patients are given in Table 2. Only two patients showed elevated γ-GT levels > 1.5 ULN, while total bile acid levels were elevated in all patients in whom it was determined (16 out of 21; range, 1.7-17.3 ULN). Three patients had a previous history of ICP; three pregnancies were twin pregnancies, and one patient experienced cholestasis under previous oral contraception.

Characteristics of patients with CIC are given in Table 3. One patient showed elevated γ-GT levels. Total bile acid levels were elevated in all three patients in whom it was determined (three out of four; range, 1.6-22.3 ULN). Oral contraceptive preparations used in the four patients contained comparable amounts of ethinylestradiol (20-35 μg) while the progesterone-like portion ranged from 50 to 150 μg. All patients had a liver biopsy done for strictly diagnostic reasons, which showed intrahepatic cholestasis in three patients. One patient had a previous history of ICP.

**Table 2 New group of patients with ICP (ICP$_{new}$)**

| Patient ID | Age (yr) | Liver parameters | | | | | Comments | | Genotypes of SNPs | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | ALT (ULN) | AP (ULN) | γ-GT (ULN) | tBili (ULN) | tBA (ULN) | No of preg/ No ICP | Others | *ABCB11* 1331T>C (V444A) | *ABCC2* 3600T>A (V1188E) | *ABCC2* 4581G>A (C1515Y) |
| 1 | 36 | 0.9 | 2.3 | 3.3 | 0.8 | 10.7 | 2/1 | | CC | TA | GA |
| 2 | 31 | 1.6 | 2.5. | 0.3 | 0.8 | 17.3 | 3/2 | | TC | TA | GA |
| 3 | 35 | 8.9 | 2.3 | 1 | 0.5 | 10 | 1/1 | | TC | TT | GG |
| 4 | 29 | 1.2 | 3 | 0.6 | 0.5 | 7 | 2/1 | | CC | TT | GG |
| 5 | 42 | 11.6 | 1.4 | 0.4 | 0.8 | 6 | 1/1 | | TC | TT | GG |
| 6 | 28 | 4.8 | 1.8 | 0.2 | 0.8 | 4.7 | 2/1 | Twins | CC | TA | GA |
| 7 | 32 | 11.9 | 3 | 1.2 | 0.9 | 3.6 | nd | | CC | TT | GG |
| 8 | 16 | 5.2 | 2 | 0.4 | nd | 3.3 | nd | | TC | TT | GG |
| 9 | 38 | 6.2 | 1.2 | 1.1 | 0.4 | 3 | 2/1 | | TC | TT | GG |
| 10 | 23 | 1.6 | 1.3 | 1.3 | 0.8 | 2.4 | 1/1 | Twins | TC | TT | GG |
| 11 | 30 | 0.4 | 0.9 | 0.2 | 0.5 | 2.4 | 3/1 | | TC | TT | GG |
| 12 | 22 | 0.3 | 1.8 | 0.1 | 0.5 | 2.1 | nd | | TC | TA | GA |
| 13 | 32 | 0.2 | 0.9 | 0.7 | 0.2 | 2 | 2/1 | | TT | TT | GG |
| 14 | 30 | 0.8 | 1.2 | 0.3 | 0.6 | 1.7 | 1/1 | | CC | TT | GG |
| 15 | 28 | 7.5 | 2.3 | 0.9 | 0.6 | nd | nd | | TC | TT | GG |
| 16 | 31 | 0.9 | 2.1 | 1.2 | 0.5 | 8.1 | 1/1 | | CC | TT | GG |
| 17 | 20 | 3.4 | 1.1 | 0.4 | 0.5 | 2.6 | 1/1 | | TT | TT | GG |
| 18 | 24 | 7.2 | 1.3 | 0.4 | 0.8 | nd | 1/1 | | CC | nd | nd |
| 19 | 31 | 8.9 | 1.3 | 1.2 | 1.5 | nd | 2/2 | | CC | nd | nd |
| 20 | 32 | 11 | 2.2 | 0.4 | 3.1 | nd | 3/3 | Pruritus with contraceptives | CC | nd | nd |
| 21 | 41 | 10.9 | 2.9 | 5.7 | 1.2 | nd | 1/1 | Twins | CC | nd | nd |
| Summary median (Q1; Q3) | 31 (28; 32) | 4.8 (0.9; 8.9) | 1.8 (1.2; 2.3) | 0.6 (0.4; 1.2) | 0.6 (0.5; 0.8) | 3.5 (2.4; 7.3) | | | | | |

n/a: Not available; ALT: Alanine aminotransferase; tBili: Total bilirubin; tBA: Total bile acids.

**Table 3 Characteristics of patients with oral CIC**

| Patient ID | Oral contraceptive | Age (yr) | Exposure time | Liver parameters | | | | | Comments | | Genotypes of SNPs | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | ALT (ULN) | AP (ULN) | γGT (ULN) | tBili (ULN) | tBA (ULN) | Clinical features | Histology | *ABCB11* 1331T>C (V444A) | *ABCC2* 3600T>A (V1188E) | *ABCC2* 4581G>A (C1515Y) |
| 1[1] | 30 μg ethinylestradiol/ 75 μg gestodene | 32 | nd | 4.9 | 1.7 | 1 | 10.9 | 22.3 | Jaundice | Intrahepatic cholestasis | CC | TT | GG |
| 2 | 30 μg ethinylestradiol/ 150 μg levonorgestrel | 15 | 21 d | 1 | 3 | 1 | 4.2 | nd | Jaundice, nausea, pruritus | Extensive intrahepatic cholestasis | CC | TT | GG |
| 3 | 35 μg ethinylestradiol/ 50 μg levonorgestrel | 40 | 2 yr | 3.9 | 2.8 | 3.6 | 0.5 | 1.6 | Pruritus | Bland | CC | TT | GG |
| 4 | 35 μg ethinylestradio/ 2 mg cyproteron | 34 | nd | 1 | 1.3 | nd | 2.8 | 1.6 | Jaundice | Extensive canalicular cholestasis, mild portal inflamation | CC | TA | GA |

[1]Patient exhibited previous episodes of ICP.

### *Sequence analysis*

**ABCB4 and ABCB11**: Sequence analysis in the 25 newly recruited patients with estrogen-associated cholestasis revealed no disease-associated non-synonymous mutations in *ABCB4* or *ABCB11*. Furthermore, in line with previous findings[11], no *ABCB4* polymorphism was found to be overrepresented in the ICP and CIC groups compared to pregnant women without cholestasis and healthy Caucasian individuals. All of the detected genetic variants in *ABCB11* and *ABCB4* were in Hardy Weinberg equilibrium.

In contrast, the *ABCB11* 1331T>C → V444A polymorphism was significantly more frequent in ICP and CIC patients compared to the two control groups. Specifically, the CC genotype was encountered in 57.1% of all ICP patients (ICP$_{new}$, 47.6% and ICP$_{old}$, 67.7%) and 100% of CIC patients compared to 20 and 32.2% in pregnant

**Table 4 Genotype distribution of non-synonymous *ABCB11* variant site 1331T>C in patients and controls**

| Genotype SNP | $ICP_{old}$ | | $ICP_{new}$ | | $ICP_{total}$ | | Pregnant controls | | Caucasian controls | |
|---|---|---|---|---|---|---|---|---|---|---|
| | *n* (%) | 95 % CI | *n* (%) | 95 % CI | *n* (%) | 95 % CI | *n* (%) | 95 % CI | *n* (%) | 95 % CI |
| *ABCB11* 1331T>C (V444A) | 21 (100) | | 21 (100) | | 42 (100) | | 40 (100) | | 205 (100) | |
| TT (VV) | - | - | 2 (9.5) | 0.0-22.1 | 2 (4.8) | 0.0-13.9 | 7 (17.5) | 5.7-29.3 | 38(18.5) | 13.2-23.9 |
| CC (AA) | 14 (67.7) | 46.5 -86.8 | 10 (47.6) | 26.3-69.0 | 24 (57.1) | 36.0-78.3 | 8 (20) | 7.6-32.4 | 66 (32.2) | 25.8-38.6 |
| TC (VA) | 7 (33.3) | 13.2-53.5 | 9 (42.9) | 21.7-64.0 | 16 (38.1) | 17.3-58.9 | 25 (62.5) | 47.5-77.5 | 101 (49.3) | 42.4-56.1 |
| Frequency C allele | 35 (83.3) | 67.4-99.3 | 29 (69.0) | 49.3-88.8 | 64 (76.2) | 58.0-94.4 | 41 (51.3) | 35.8-66.7 | 233 (56.8) | 50.1-63.6 |
| Frequency T allele | 7 (16.7) | 0.7-32.6 | 13 (31.0) | 11.2-50.7 | 20 (23.8) | 5.6-42.0 | 39 (48.8) | 33.3-64.2 | 177 (43.2) | 36.4-50.0 |
| *ABCC2* 3563T>A (V1188E) | 16 (100) | | 17 (100) | | 33 (100) | | 42 (100) | | 110 (100) | |
| TT (VV) | 15 (93.8) | 71.3-98.6 | 13 (76.5) | 52.3-90.4 | 28 (84.8) | 68.9-93.3 | 37 (88.1) | 74.3-96.1 | 95 (86.4) | 68.9-93.3 |
| AA (EE) | - | - | - | - | - | - | - | - | 1 (0.9) | 0.0-5.0 |
| TA (VE) | 1 (3.1) | 0.0-15.8 | 4 (23.5) | 9.6-47.7 | 5 (15.2) | 6.7-31.1 | 5 (11.9) | 3.9-25.7 | 14 (12.7) | 7.1-20.5 |
| *ABCC2* 4544G>A (C1515Y) | 16 (100) | | 17 (100) | | 33 (100) | | 42 (100) | | 110 (100) | |
| GG (CC) | 15 (93.8) | 71.3-98.6 | 13 (76.5) | 52.3-90.4 | 28 (84.8) | 68.9-93.3 | 36 (85.7) | 71.4-94.6 | 95 (86.4) | 68.9-93.3 |
| AA (YY) | - | - | - | - | - | - | - | - | 1 (0.9) | 0.0-5.0 |
| GA (CY) | 1 (3.1) | 0.0-15.8 | 4 (23.5) | 9.6-47.7 | 5 (15.2) | 6.7-31.1 | 6 (14.3) | 5.4-28.6 | 14 (12.7) | 7.1-20.5 |

Results are given with 95 percent confidence interval (95% CI).

**Table 5 1331T>C (V444A): Fisher's exact test and ORs for the presence of homozygous CC variant and the C allele in the different groups**

| | CC *vs* TT | | | C *vs* T | | |
|---|---|---|---|---|---|---|
| | Fisher | Odds ratio | 95% CI | Fisher | Odds ratio | 95% CI |
| $IC_{Pold}$ *vs* Pregnant controls | 0.0041 | nd[1] | - | 0.0004 | 4.8 | 2.2-15.0 |
| $IC_{Pold}$ *vs* Caucasian controls | 0.0029 | nd[1] | - | 0.0005 | 3.8 | 1.9-11.1 |
| $IC_{Pnew}$ *vs* Pregnant controls | 0.1082 | 4.4 | 0.7-27.2 | 0.0441 | 2.1 | 1.0-4.7 |
| $IC_{new}$ *vs* Caucasian controls | 0.1461 | 2.9 | 0.6-13.8 | 0.0850 | 1.7 | 0.9-3.4 |
| $IC_{Ptotal}$ *vs* Pregnant controls | 0.0065 | 10.5 | 1.9-63.7 | 0.0007 | 3.0 | 1.7-6.4 |
| $IC_{Ptotal}$ *vs* Caucasian controls | 0.0025 | 6.9 | 1.6-32.1 | 0.0006 | 2.4 | 1.5-4.5 |

[1]Could not be determined, as TT = 0.

women without cholestasis and healthy Caucasian controls, respectively (Table 4). In line with these findings, the ORs of C *versus* T were 3.0 (1.7-6.4) for all ICP patients ($ICP_{new}$ + $ICP_{old}$) *versus* healthy pregnant control women ($ICP_{new}$, 2.1; 1.0-4.7 and $ICP_{old}$ 4.8; 2.2-15.0) (Table 5 and Figure 1). With the exception of this polymorphism and two intronic variants that were found to be closely linked to the 1331T>C polymorphism in previous studies [intron 13: (+70) C>T and intron 14 (+32) T>C][26], the allele frequency of the remaining common variants in the patients with ICP and CIC was comparable to that observed in healthy pregnant and Caucasian controls. Due to the small sample size, no significance levels could be calculated for the CIC group. However, all patients in this group were homozygous for the C at position 1331, which is highly suggestive of an overrepresentation of this allele compared to the control groups.

**ABCC2:** The 1249G>A polymorphism was not found in our patients. In contrast, 3563T>A and 4544G>A were strongly linked and distributed similarly in all groups (Table 4). No significant difference in the frequency of these two polymorphisms was observed between affected ICP and CIC patients and healthy controls. Heterozygous carriers of the 3563A and the 4544A alleles were found in 15.2% of ICP patients ($ICP_{new}$, 23.5% and $ICP_{old}$, 3.1%) compared to 11.9 and 12.7% in pregnant women without cholestasis and healthy Caucasian controls, respectively (Table 4). Furthermore, one CIC patient was a heterozygous carrier for the two variant alleles at positions 3563 and 4544.

### *Relation of serum bile acid levels and the ABCB11 1331T>C genotype*

For correlation of bile acid levels with the corresponding genotype at position 1331 of *ABCB11*, ICP and CIC samples were analyzed together. Bile acid levels were available for 16 out of 21 $ICP_{new}$ patients, seven out of 20 $ICP_{old}$ patients, and three out of four CIC patients, which yielded a total of 26 samples (CC, 14 patients; CT, 10 patients; and TT, two patients). Interindividual variability in serum bile acid levels was high and ranged from 1.7 to 22.3 ULN and 1.7 to 17.3 ULN in CC and CT patients, respectively. Serum bile acid levels gradually increased from carriers of the TT genotype to carriers of the CC genotype, with medians of 2.3 ULN (Q1, 2.2; Q3, 2.5), 3.2 ULN (Q1, 2.4; Q3, 6.2) and 4.4 ULN (Q1, 2.2; Q3, 7.8) for TT, TC and CC, respectively (Figure 2).

## DISCUSSION

We investigated the risk association between different *ABCB11* and *ABCC2* polymorphisms in ICP and CIC, and correlated different genotypes with serum bile acid levels

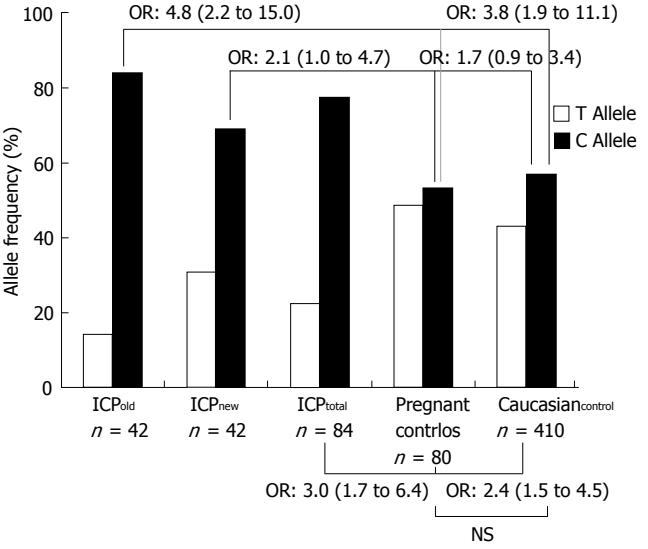


**Figure 1** Allelic frequency of the T allele (white panel) and C allele (black panel) of the ABCB11 1331T>C (1331T>C) polymorphism. 21 $ICP_{new}$ patients (42 alleles); 21 $ICP_{old}$ patients (42 alleles); 42 $ICP_{total}$ patients (84 alleles); 20 $ICP_{control}$ patients (40 alleles); 205 $Caucasian_{control}$ patients (410 alleles). Allelic frequencies and ORs are given with 95% CI. Groups were compared with Fisher's exact test.

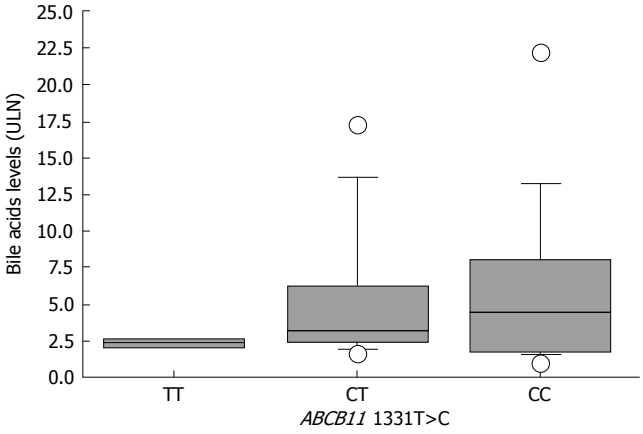


**Figure 2** Bile acid levels in patients harboring different 1331C>T genotypes. TT: two patients; CT: 10 patients; CC: 14 patients.

as a marker of cholestasis. In our group of 25 patients with estrogen-associated cholestasis (21 with ICP and four with CIC), there was a highly significant association between the presence of the C allele at position 1331 of *ABCB11* and the presence of cholestasis, which confirms preliminary results from another collective of ICP women, in whom such an overrepresentation was first observed[11]. While *in vitro* function of both BSEP variants, as measured by taurocholate transport activity, is comparable[24], BSEP expression in healthy liver tissue of Caucasian individuals has recently been found to be lower in carriers of the 1331C allele[25]. Such differences in hepatic BSEP expression levels might offer one valuable explanation for the increased susceptibility to the development of cholestasis under specific circumstances, such as hormonal challenges. Furthermore, serum bile acids as a marker of *in vivo* BSEP function was influenced by the underlying genotype. Lowest bile acid levels were observed in patients with the TT genotype and highest levels in carriers of the CC genotype. Although the accepted level of statistical significance was not reached due to high interindividual variability, this observation is in line with the hypothesis that the underlying genotype at position 1331 is a determinant of BSEP function, and hence contributes to the individual risk of developing cholestasis.

The homozygous state for the 1331T>C polymorphism has only recently been observed in a very severe case of pregnancy-associated cholestasis with serum bile acid levels > 40-fold above the ULN. Interestingly, decreased BSEP expression levels were found in a liver biopsy obtained from this patient[27]. Although this patient carried an additional *ABCB4* mutation, the presence of decreased hepatic BSEP expression and highly elevated bile acid levels strongly support a BSEP-related mechanism as a predominant pathogenetic factor. The same patient also developed severe cholestasis under previous use of oral contraceptives, which supports the notion that the same polymorphism also predisposes to oral CIC.

In our group, seven patients (from $ICP_{old}$) carried additional *ABCB4* mutations, while no such mutations were detected in the remaining 35 out of 42 ICP patients ($ICP_{old}$, 14 and $ICP_{new}$, 21). This finding suggests that the *ABCB11* 1331T>C polymorphism independently contributes to an individual's risk for developing cholestasis under certain conditions. On the other hand, it can be speculated whether the combination of the 1331T>C polymorphism with *ABCB4* mutations might be a risk constellation for a severe disease course, as observed by Keitel and coworkers[27].

In contrast, no association was found between the presence of the non-synonymous polymorphisms at positions 1188 and 1515 of MRP2 and the presence of ICP or CIC. A possible pathogenic role of these two polymorphisms in ICP and CIC was suspected based upon the genotype-dependent alteration in hepatic MRP2 expression levels in healthy human liver tissue[25]. Specifically, heterozygous carriers of the glutaminic acid at position 1188 and tyrosine at position 1551 showed significantly higher levels of MRP2 in their liver than homozygous carriers of valine and cysteine, respectively[25]. As BSEP inhibition by estrogen and progesterone metabolites requires prior MRP2-mediated secretion into the bile canaliculus, high MRP2 expression was suspected as a risk factor for the development of estrogen-dependent cholestasis[22].

Several conclusions can be drawn from this study. First, our data point toward a pathogenic relevance of the *ABCB11* 1331T>C polymorphism in ICP and CIC. While these types of cholestasis are so far mainly attributed to different disease-causing mutations in *ABCB4*[5,11,12], our data support a clear association between the presence of a frequent *ABCB11* polymorphism and ICP. Interestingly, all of the patients with CIC were homozygous carriers of the C allele at position 1331. It can be speculated that lower estrogen levels in CIC compared to second or third trimester pregnancy require two low-function alleles to result in cholestasis. Furthermore, the 1331T>C variant was also found to be associated with other inherited and acquired forms of cholestasis, such as benign recurrent intrahepatic cholestasis and drug-induced cholestasis[24,28,29]. This suggests a role for this polymorphism as a risk factor for different cholestatic conditions, which have so far been regarded as different disease entities[20,30].

Second, while γ-GT levels are elevated in ICP patients who carried a disease-causing *ABCB4* mutation[11], serum bile acid levels are influenced by the BSEP genotype at position 444 of *ABCB11*. It can, therefore, be speculated that these two parameters allow us to clinically distinguish between MDR3- and BSEP-related forms of estrogen-related cholestasis, as it is already done for progressive forms of inherited familial intrahepatic cholestasis[5,21]. From a prognostic point of view, this might help to distinguish patients that carry a common susceptibility factor from those who carry a disease-causing *ABCB4* mutation, which in some cases, has been associated with disease progression[7,11,12,31]. Third, although a pathogenic involvement of MRP2 in estrogen-induced cholestasis has longly been suspected, common *ABCC2* polymorphisms have not been associated with the development of cholestasis. We did not exclude the presence of disease-associated *ABCC2* mutations in our group, but normal bilirubin levels in all but one patient suggests no major MRP2 dysfunction, which should result in a Dubin Johnson phenotype[32].

In summary, our data support a role for the *ABCB11* 1331T>C polymorphism as a susceptibility factor for the development of estrogen-induced cholestasis, whereas no such association was found for *ABCC2*. Serum bile acid and γ-GT levels might help to distinguish *ABCB4* and *ABCB11*-related forms of ICP and CIC.

## COMMENTS

### Background

Intrahepatic cholestasis of pregnancy (ICP) and oral contraceptive-induced cholestasis (CIC) are two acquired forms of cholestasis, which are observed in otherwise healthy young women with a normal medical history. The bile salt export pump (BSEP, *ABCB11*) and the multidrug resistance protein 2 (MRP2, *ABCC2*) might be of pathogenetic importance in both conditions.

### Research frontiers

A genetic predisposition for both types of hormonal cholestasis has been suspected based upon the strong regional clustering, the higher prevalence in female family members of patients with ICP, and the co-incidence with hereditary cases of progressive familial intrahepatic cholestasis. While mutations in the *ABCB4* gene that encodes the canalicular phospholipid flippase multidrug resistance protein 3 (MDR3) have been implicated in the development of ICP and CIC in a subset of affected patients, the role of genetic variants in *ABCB11* and *ABCC2* remains unclear.

### Innovations and breakthroughs

Our data support a role of the *ABCB11* 1331T>C polymorphism as a susceptibility factor for the development of estrogen-induced cholestasis, whereas no such association was found for *ABCC2*. Serum bile acid and γ-GT levels might help to distinguish *ABCB4*- and *ABCB11*-related forms of ICP and CIC.

### Applications

While the clinical consequences of such findings are still uncertain at this time, they provide important new insights in the role of genetically determined differences in canalicular transporter expression and function for the development of estrogen-induced cholestasis. In the future, the integration of different factors that predict cholestasis might be used to counsel pregnant patients or to avoid certain medications in susceptible patients.

### Terminology

ICP: Intrahepatic cholestasis of pregnancy; CIC: contraceptive-induced cholestasis; BSEP: Bile Salt Export Pump (*ABCB11*); MRP2: Multidrug Resistance Protein 2 (*ABCC2*); MDR3: Multidrug Resistance Protein 3 (*ABCB4*).

### Peer review

The study characterized a potential underlying defect in the subgroup of normal γ-GT ICP patients and contributes to a clinical risk assessment for the future. This study from a group with longstanding experience in transporter genomics is well designed and presented in a clearly written manuscript.

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*CLINICAL RESEARCH*

# Factors that impact health-related quality of life in adults with celiac disease: A multicenter study

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## Abstract

**AIM:** To evaluate the factors involved in the impairment of health-related quality of life (HRQOL) in patients with celiac disease.

**METHODS:** A multicenter, cross-sectional prospective study was performed in patients with celiac disease who completed two HRQOL questionnaires: the gastrointestinal quality of life index (GIQLI) and the EuroQol-5D (EQ).

**RESULTS:** Three hundred and forty patients (163 controlled with a gluten-free diet, and 177 newly diagnosed with a normal diet) were included. The GIQLI score was significantly better in patients on a gluten-free diet (GFD) than in non-treated patients on their usual diet, both in terms of the overall score (3.3 *vs* 2.7, respectively; $P < 0.001$), as well as on the individual questionnaire dimensions. Both the preference value of the EQ as the visual analogue scale were significantly better in treated than in non-treated patients (0.93 *vs* 0.72 $P < 0.001$ and 80 *vs* 70 $P < 0.001$, respectively). Variables significantly associated with a worse HRQOL score were female gender, failure to adhere to a GFD, and symptomatic status.

**CONCLUSION:** In untreated celiac disease, the most important factors that influence patient perception of health are the presence of symptoms and a normal diet. HRQOL improves to levels similar to those described in the general population in celiac disease patients well controlled with a GFD.



**Key words:** Celiac disease; Health status; Quality of life; Gluten-free diet

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## INTRODUCTION

Celiac disease (CD) is a chronic immuno-inflammatory enteropathy that appears in genetically predisposed patients. Patients with this disease develop characteristic small-intestinal-mucosal changes, due to hypersensitivity to gluten. Several epidemiological factors, including a dramatic increase in the diagnosis of CD[1] and changes in its presentation, with the appearance of atypical forms, that may even mimic functional diseases, as well as the fact that it affects people of any age[2] mean that CD is currently considered a common and important health-care problem. It affects approximately 1%-2% of adults in Western European populations[3].

Although the understanding of the immunology and physiopathology of CD is extensive[4,5], the impact of the disease from the patient's point of view has received less attention. The chronic nature of the condition, together with the limitations imposed by the need to follow a permanent restrictive diet, substantial numbers of physician visits, the risk of associated diseases and potential complications, mean that CD can have a considerable negative impact on health-related quality of life (HRQOL). Due to the lack of disease-specific instruments to measure HRQOL in CD patients, generic questionnaires, such as the SF-36 or EuroQol-5D (EQ), have been used. Overall perception of HRQOL is rated

as bad or fair by 63% of patients before diagnosis of CD, and improves to 77% after treatment[6]. However, at least one study has indicated that treated CD patients rate their overall well-being, as measured with a visual analog scale (VAS), quite highly, with 83.6% of patients being rated as 'very well' or 'well'[7]. Apart from dietary treatment, other factors have been suggested to influence the HRQOL in CD patients. Some disease-related factors, such as the presence of symptoms or associated co-morbidity, as well as the type of diagnosis used (symptom-detected *vs* screening-detected), have also been shown to impair CD patients' HRQOL[8,9]. Non-disease-related factors, such as female gender, also have a negative impact on HRQOL[10], although this is also true in other chronic diseases[11]. The impairment of HRQOL in untreated celiac disease and its improvement after treatment with a gluten-free diet (GFD), has been confirmed using generic multidimensional HRQOL measures[12]. However, there is no agreement as to whether the HRQOL of treated CD patients is similar[8,13,14] or not[15,16] to that of the general population.

The aims of the present study were to measure and compare the HRQOL in treated and untreated CD patients using condition-specific and generic measures of HRQOL, and to analyze the factors impacting on HRQOL in these patients. A further objective was to compare scores on the generic measure of HRQOL for the two study groups with general population reference scores for the same instrument (EQ). To this end, we performed a multicenter study in a representative sample of patients from different parts of Spain, including both large and small cities, and both island and mainland regions.

## MATERIALS AND METHODS

### Study subjects

Subjects were adult outpatients with CD who attended the Digestive Services Units of seven different Spanish hospitals, over a one-year period. Diagnosis was based on current serological and histological criteria[17]. A total of 340 CD patients were included. At the time of inclusion, patients were stratified into two groups, according to whether they were already following a GFD (GFD group) or whether they had not yet started on the GFD (pretreatment group).

### Procedure

All patients completed a questionnaire on demographic details, current symptoms, information on the disease, and issues related to the GFD. They also completed the Gastrointestinal Quality of Life Index (GIQLI) questionnaire and the EQ, two generic HRQOL instruments.

Patient compliance following the GFD was measured using an adapted version of the self-administered questionnaire developed by Morisky *et al*[18]. The questionnaire consisted of four items which measure the degree of treatment compliance, answered using dichotomous response options (yes/no). Two questions asked about unintentional lack of compliance ("sometimes I forget my diet/sometimes I do not comply carefully with my diet"), while the other two questions dealt with intentional lack of compliance ("when I feel well I sometimes discontinue my diet/when not feeling well I sometimes discontinue my diet"). If either question 3 or 4 was answered affirmatively, the patient was considered to have voluntarily discontinued his or her diet. If either question 1 or 2 was answered in the affirmative, the patient was considered to have involuntarily neglected or forgotten his or her diet. This questionnaire was originally developed to measure compliance with medication, and was adapted for use in the current population by substituting drugs with GFD. In a previous study[2], many patients suggested that they never forgot about their diet; for that reason, a fifth option was added to the scale ("I never forget about my diet"). Patients checking this answer were considered good compliers.

In an ancillary study, 17 patients from the pre-treatment group completed the questionnaires both at inclusion, when they had not yet started on the GFD, and at least 6 mo following treatment with GFD.

### HRQOL assessment

HRQOL was assessed using two generic questionnaires. GIQLI is a self-administered questionnaire designed to assess HRQOL in patients with gastrointestinal diseases. It was chosen because it has been translated and validated for use in Spain[19]. The GIQLI consists of 36 items grouped into five domains of health (gastrointestinal symptoms, physical dysfunction, social dysfunction, emotional dysfunction and treatment effects). Responses are scored on a 4-point Likert scale, in which 4 corresponds to the highest level of functioning. The instrument produces five dimension scores and an overall score ranging from 0 to 4, with a higher score reflecting better HRQOL.

EQ is a short self-administered generic utility measure that provides both a descriptive profile and an overall index for HRQOL. The EQ includes five dimensions (mobility, personal care, daily activities, pain and anxiety-depression) answered on a 3-point scale ranging from no problems (level 1) to extreme problems (level 3). Combining one level of severity from each of the five dimensions generates a number of discrete health states that can be assigned preference values ranging from 0, which represents worst health status, to 1 (best health status). The EQ also includes a VAS that ranges from 0 (worst imaginable health status) to 100 (best imaginable health status), on which patients were asked to mark the point that best reflected their health status on the day of the interview. The EQ has also been translated and validated in Spanish[20]. As a reference, normal values for a representative sample of 12245 members of the Spanish general population also exist for the EQ, and we used these as a control for the patients included in the present study[21,22].

### Statistical analysis

The Kolmogorov-Smirnov test showed that most of the study variables did not have a normal distribution. Descriptive analyses of socio-demographic, clinical and HRQOL data were therefore performed using medians and 25$^{th}$ and 75$^{th}$ percentiles. Qualitative variables were described as proportions. Comparisons between variables were performed using the Mann-Whitney *U*, Kruskal-Wallis or Fisher tests as appropriate. In the pre-treatment group, HRQOL was also analyzed depending on whether the diagnosis was performed on the basis of symptoms or on positive serology.

**Table 1 Socio-demographic and clinical characteristics of patients *n* (%)**

| | Pre-treatment | GFD |
|---|---|---|
| Number | 177 | 163 |
| Age (yr) | 44 (30-50) | 37 (28-47)[a] |
| Sex (male/female) | 74/103 | 42/121 |
| Smoking status | | |
| Smoker | 29 (16) | 33 (25) |
| Non-smoker | 148 (84) | 130 (75) |
| City of residence | | |
| < 100 000 inhabitants | 69 (43) | 61 (39) |
| 100 000-500 000 | 72 ( 45) | 65 (41) |
| > 500 000 | 18 (12) | 31 (20) |
| Family status | | |
| Single | 57 (33) | 67 (42) |
| Married | 108 (63) | 84 (53) |
| Widowed | 5 (4) | 8 (5) |
| Educational level | | |
| No studies | 7 (4) | 4 (2) |
| Primary | 56 (33) | 38 (24) |
| Secondary | 55 (32) | 61 (38) |
| University studies | 52 (31) | 55 (36) |
| Occupational status | | |
| Employee/self-employed | 67 (41) | 94 (62) |
| Retired/pensioner | 36 (22) | 14 (9) |
| Housewife | 30 (18) | 16 (10 ) |
| Student | 12 (7) | 19 (12) |
| Unemployed | 17 (12) | 10 (7) |
| Disease duration (mo) | - | 48 (24-84) |
| Duration of symptoms before diagnosis (mo) | 24 (7-84) | 12 (5-40) |
| Presence of symptoms at inclusion | 118 (80) | 52 (33)[b] |
| Presence of associated diseases at inclusion | 42 (41) | 41 (37) |

Results are expressed as medians and (25th and 75th percentiles) or absolute values and (percentage). [a]$P < 0.05$, [b]$P < 0.01$.

Three multiple linear regression models were developed to identify independent variables that influence HRQOL. Dependent variables in the three models were the overall GIQLI score, the EQ preference value index, and the EQ VAS scores. Independent variables in all models were GFD, sex, age, duration of symptoms, and the binary variables were existence of symptoms and presence of CD-associated diseases. Results obtained for the multiple linear regression model were the regression coefficient, *t*-test statistics, *P* values, and the variance inflation factor. The *t* test established whether the independent variables contributed to predicting the dependent variables. Higher *t*-test values indicate that the independent variable more strongly predicts the dependent variable. It has been considered that each independent variable contributes to predicting the dependent variable when $P < 0.05$. The variance inflation factor, a measure of multicollinearity, measures the inflation of the standard error of each regression coefficient for an independent variable due to redundant information in other independent variables. If the variance inflation factor is 1.0, there is no redundant information in the other independent variables. The level of statistical significance for the multiple regression model was set at $P < 0.05$.

## RESULTS

### Patient and disease characteristics

A total of 340 patients from seven hospitals in different areas of Spain were included. Table 1 summarizes the socio-demographic and clinical characteristics, according to whether they were in the GFD group ($n = 163$) or the pre-treatment group ($n = 177$).

There were no relevant differences in demographic characteristics between the two groups. Age ranged from 15 to 78 years. Treated patients were younger than non-treated patients [median age 37 (28-47) *vs* 44 (30-50) years, respectively; $P < 0.05$], but the difference was not considered clinically relevant. The sample characteristics were also in accordance with the results of previous epidemiological studies of CD in Spain[2], with patients being young adults and predominantly female and non-smokers. Most patients lived in small to medium-sized cities, with < 500 000 inhabitants.

The duration of disease since diagnosis was not evaluated in the normal diet group because they were included at the time of diagnosis. There were no statistically significant differences in duration of symptoms before diagnosis of CD between treated and pre-treated patients. The disease presented in the classic form in 47 (32%) patients in the GFD group and in 13 (11%) patients in the normal diet group ($P < 0.01$). The most frequently presenting non-classical forms of CD included anemia (32%), cutaneous lesions (9%), and hypertransaminasemia (6%). Approximately 40% of patients had associated diseases, such as thyroid diseases (7%), selective IgA deficiency (3%), type I diabetes mellitus (5%), depression (11.1%), and chronic inflammatory arthropathy (8%). The percentage of asymptomatic patients at inclusion was significantly higher in the treated GFD group than in the normal diet group (80% *vs* 33%, $P < 0.01$). In the GFD group, 117 patients (73%) were classified as good compliers on the basis of their Morisky scores, 36 (22%) reported that they occasionally involuntarily neglected or forgot the diet, and eight (5%) had voluntarily discontinued the diet. Again in the GFD group, 47% of patients reported complete disappearance of symptoms since being on the diet, and 43% reported a significant improvement in symptoms. Eight per cent of patients reported a small improvement in symptoms, and 2% considered that their symptoms had not changed with treatment.

### Description of HRQOL in CD

Figure 1 shows the overall score and the five dimensions of GIQLI for the GFD and pre-treatment groups. Patients in the GFD group reported significantly ($P < 0.001$) better HRQOL than patients in the pre-treatment group for the overall score and the gastrointestinal symptoms, and in the physical, social and emotional dysfunction dimensions.

Patients in the pre-treatment normal diet group scored best on GIQLI treatment effects domain [4.0 (3.0-4.0)], while the domains with the worst scores were physical dysfunction [2.3 (1.4-2.9)], emotional dysfunction [2.4 (1.8-3.0)], and gastrointestinal symptoms [2.8 (2.4-3.3)]. These results suggested that most impaired domains of health in newly diagnosed CD patients were related to symptomatic and emotional dimensions. In GFD patients, the best scored domain was also the treatment domain [4.0 (3.0-4.0)] and the worst scored was emotional dysfunction [3.0 (2.4-3.4)], which

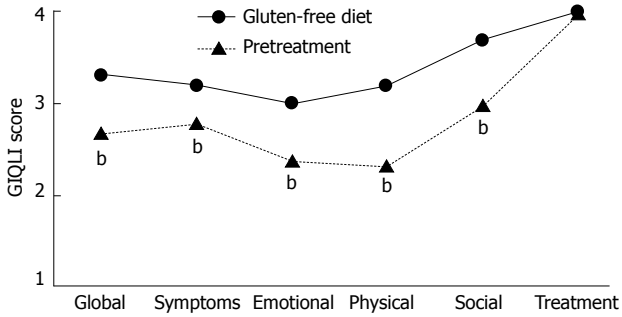


**Figure 1** Median global and dimension scores of GIQLI for GFD (circles) and untreated (triangles) patients. Scores were significantly higher ($P < 0.001$) for all dimensions except treatment. [b]$P < 0.001$.

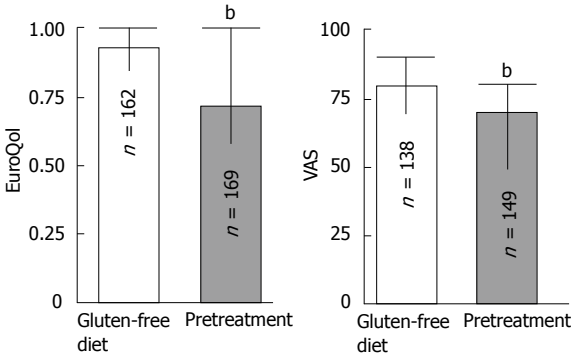


**Figure 2** Median scores and 25th and 75th percentiles for the tariff and VAS of the EQ for GFD (white columns) and normal diet (grey columns). Scores were significantly better ($P < 0.001$) for patients in the GFD group. [b]$P < 0.001$.

suggested that, although improved by treatment, the most sustained impairment affected the emotional dimension.

Median scores on the EQ preference value index and VAS are shown in Figure 2. The median EQ preference value was significantly higher in GFD patients than in the normal diet group [0.93 (0.85-1.0) *vs* 0.72 (0.58-1.0) respectively, $P < 0.001$]. The median VAS score was also significantly higher in GFD patients than in the normal diet group [80 (70-90) *vs* 70 (50-80) respectively, $P < 0.001$]. Presence (levels of severity 2 and 3) or absence (level 1) of problems for each EQ dimension for the GFD and normal diet patients are shown in Table 2. At diagnosis, the normal diet group patients reported problems with significantly greater frequently than those in the GFD group on all five dimensions of the EQ, which confirmed that GFD improved patient perception of health. In the normal diet group, problems were reported most frequently in the pain/discomfort (62%) and anxiety/depression (54.5%) dimensions, and least frequently in the self-care dimension (10.2%). In the GFD group, problems were reported most frequently on the EQ anxiety/depression dimension (33.9%), in consonance with the low score of the emotional dimension in the GIQLI observed in this group of patients.

### *Factors influencing HRQOL in CD*

Table 3 shows the results of the multiple regression analysis, which took the GIQLI overall score as a dependent variable for all patients. The analysis suggested

**Table 2 Description of presence or absence of problems for each EQ dimension according to treatment *n* (%)**

| Variable | Pre-treatment | | Gluten-free diet | |
|---|---|---|---|---|
| | No problems | Some problems | No problems | Some problems |
| Mobility | 130 (74.3) | 45 (25.7) | 146[b] (90.1) | 16 (9.9) |
| Self-care | 158 (89.8) | 18 (10.2) | 161[b] (99.3) | 1 (0.7) |
| Usual activities | 113 (64.5) | 62 (35.5) | 135[b] (83.3) | 27 (16.7) |
| Pain/Discomfort | 67 (38) | 109 (62) | 117[b] (72.8) | 45 (27.7) |
| Anxiety/Depression | 80 (45.5) | 96 (54.5) | 107[b] (66.1) | 55 (33.9) |

[b]$P < 0.001$.

**Table 3 Results of the multiple regression modeling with global GIQLI score as a dependent factor**

| Independent variable | *t*-test statistics | *P* value | Variance inflation factor |
|---|---|---|---|
| Pretreatment/treatment | 4.989 | < 0.001 | 1.31 |
| Age | -0.725 | 0.469 | 1.31 |
| Gender (m/f) | -2.684 | 0.008 | 1.06 |
| Smoking habit | 1.038 | 0.301 | 1.02 |
| Symptoms duration | -1.813 | 0.072 | 1.08 |
| Presence of symptoms | -4.932 | < 0.001 | 1.31 |
| Associated diseases | -1.824 | 0.070 | 1.18 |

**Table 4 Results of the multiple regression modeling with the preference value of the EQ score as the dependent factor**

| Independent variable | *t*-test statistics | *P* value | Variance inflation factor |
|---|---|---|---|
| Pretreatment/treatment | 3.20 | 0.002 | 1.31 |
| Age | 0.44 | 0.663 | 1.20 |
| Gender (m/f) | -1.12 | 0.261 | 1.07 |
| Smoking habit | 0.58 | 0.559 | 1.02 |
| Symptoms duration | -1.61 | 0.109 | 1.08 |
| Presence of symptoms | -5.09 | < 0.001 | 1.31 |
| Associated diseases | -2.61 | 0.01 | 1.18 |

that the presence of symptoms, normal diet and female gender were the strongest determinants ($P < 0.001$) for a worse perception of HRQOL.

Similar results were obtained using the linear regression analysis with the EQ preference value index (Table 4) and the EQ VAS (Table 5) as the dependent variables, and included the same independent variables as described earlier. Presence of symptoms and normal diet represented significant determinants of worse HRQOL. In this case, the presence of CD-associated diseases was also associated with poorer HRQOL.

In the pre-treated normal diet group, results of the comparison of patients diagnosed according to the presence of symptoms or a positive serology ($n = 26$) are shown in Figure 3. HRQOL was significantly worse in patients diagnosed through symptoms rather than through serologic tests without observable symptoms. The worst HRQOL was, therefore, found in women who had been recently diagnosed due to the presence of symptoms, who were in the normal diet group, and who had associated diseases.

In the GFD group, specific potential factors that may be related to HRQOL, such as compliance or response to

**Table 5 Results of the multiple regression modeling with the VAS of the EQ score as a dependent factor**

| Independent variable | *t*-test statistics | *P* value | Variance inflation factor |
|---|---|---|---|
| Pretreatment/treatment | 2.97 | 0.003 | 1.37 |
| Age | 0.81 | 0.420 | 1.23 |
| Gender (m/f) | -1.48 | 0.141 | 1.11 |
| Smoking habit | -0.49 | 0.624 | 1.02 |
| Symptoms duration | -2.71 | 0.007 | 1.08 |
| Presence of symptoms | -5.99 | < 0.001 | 1.40 |
| Associated diseases | -2.50 | 0.013 | 1.17 |

diet, were assessed. There were no statistically significant differences in median overall GIQLI score between patients classified as good compliers ($n$ = 117), and those who involuntarily neglected or forgot their diet ($n$ = 36), and those who had voluntarily interrupted their diet ($n$ = 8) [median GIQLI (IQR) scores, 3.3 (2.8-3.5), 3.2 (2.8-3.4), and 2.9 (2.6-3.3), respectively]. In contrast, GIQLI scores were significantly higher (better HRQOL) in the 73 patients who reported achieving complete control of symptoms with the GFD, than in patients reporting a partial or non-response ($n$ = 84) [3.4 (3.1-3.6)] *vs* [3.0 (2.7-3.4), $P < 0.001$]. With reference to the potential effect of GFD duration on HRQOL, the duration of treatment and GIQLI overall score were slightly correlated ($r = 0.19$, $P < 0.05$) according to the Spearman rank correlation test. Similar correlation was observed with the EQ preference value index and the EQ VAS ($r = 0.20$, $P < 0.05$ and $r = 0.15$, $P$ = NS).

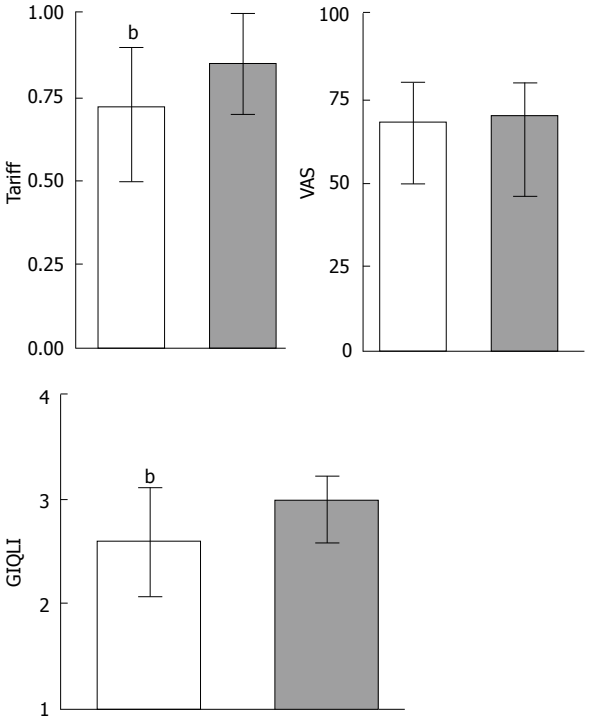


**Figure 3** Median scores and 25th and 75th percentiles for the global GIQLI (left panel) and EQ index and VAS (right panel) in the pre-treated normal diet CD patients (white columns represents patients diagnosed due to symptoms and grey columns those diagnosed by serological tests). Scores were significantly worse ($P < 0.05$) for patients diagnosed due to symptoms compared to those with isolated serologic tests. [b]$P < 0.001$.

### *Intensity of impairment of HRQOL in CD*

Although statistical comparisons between CD patients' EQ scores and those described for the Spanish general population (median preference value of 1.0 and VAS score of 80) were not performed, it seems apparent that treated patients do not have different preference values and VAS scores to the Spanish general population. In contrast, preferences and VAS scores of the normal diet group were worse than those of the general population. According to the distribution of EQ VAS scores in the Spanish general population (21), CD patients perceive their health as good to excellent (VAS scores between 70 and 100), while untreated patients with newly diagnosed CD have VAS scores corresponding to fair health.

### *Changes in HRQOL of CD before and after treatment*

Seventeen newly diagnosed patients [11 women, six men, median age 48 (34-60) years] completed the HRQOL questionnaire before initiating treatment and after 6-23 mo with GFD. Paired comparisons using the Wilcoxon signed rank test showed that treatment improved significantly the GIQLI overall score [from a median of 2.6 (2.3-3.0) to 2.9 (2.7-3.4), $P$ <0.001] and the VAS score [from 70 (55-70) to 77 (70-87) respectively, $P < 0.01$]. However, the median EQ preference value increase after treatment did not reach statistical significance [0.85 (0.69-1.0) to 0.87 (0.71-1.0), $P$ = NS].

## DISCUSSION

To analyze how CD affects HRQOL and to determine which factors are associated, we administered two generic instruments to measure HRQOL in two groups of CD patients: 163 patients following a GFD and 177 newly diagnosed patients who were on a normal diet. A potential limitation of the study is a selection bias for patients because we only included hospital-controlled patients. Results on the GIQLI and EQ questionnaires suggest that the HRQOL of untreated recently diagnosed CD patients is significantly impaired, on almost all of the dimensions on both instruments and on the overall score. It is not surprising then that these patients' assessment of their overall health on the EQ should be equivalent to a rating of only fair health. Patients before starting a GFD only scored well on the treatment effects dimension on the GIQLI questionnaire. Although these patients have not initiated the GFD, the highly-scored dimension was treatment, probably because this item refers to "feeling let down by treatment effects" and most patients have received prior diet or drug recommendations, or because patients tend to respond neutrally to items in this domain. By contrast, CD patients controlled with a GFD reported significantly better HRQOL, and in fact had EQ scores that were very similar to those of a representative sample of the Spanish general population[21,22]. These results study are in line with those reported very recently by the Canadian Celiac Association[23]. In that study, it was found that SF-12 summary scores for CD patients were similar to the normative Canadian data, except for females and newly diagnosed patients.

The importance of adequate compliance with a GFD is confirmed by the multivariate analysis, in which it was shown that GFD exerted a major influence on HRQOL,

whether using the condition-specific GIQLI or the generic EQ instrument. To avoid the problem of between-person variability in the determination of the effect of GFD on HRQOL, in a subgroup of newly diagnosed patients, the HRQOL was re-measured after at least 6 mo on GFD. Results of that ancillary study confirmed that treatment of CD improves HRQOL. The multivariate analysis also indicated that the presence of symptoms and comorbidity were other independent variables that significantly influenced HRQOL in CD patients. These results suggest that compliance with GFD and adequate control of symptoms are decisive, in terms that CD patients may recover to satisfactory levels of self-perceived health. The duration of improvements in HRQOL in CD patients following a GFD could not be deduced from our study, but it has been suggested that such improvements may be maintained for as long as 20 years[14].

Although our study confirmed that a GFD improves symptoms, with 90% of patients reporting a clinically significant improvement, as well as raising CD patients' levels of HRQOL to those reported by the general population, other studies, such as that by the Westchester Celiac Sprue Support Group have observed negative effects of a GFD on HRQOL in areas such as dining out, travel and family life[24]. These aspects were not covered in depth by either of the HRQOL instruments included here, and they should be considered for inclusion in future studies.

Advances in the serologic diagnosis of CD mean that the disease is recognized at increasingly early times, even in asymptomatic patients. Presumably as a result of the absence of symptoms in patients diagnosed by serologic screening, as opposed to patients diagnosed by clinical symptoms, it has been suggested that screening-detected CD patients have better HRQOL than those diagnosed based on symptoms[10,25]. There is no agreement as to whether a GFD improves HRQOL independently of whether diagnosis was based on serologic screening or on symptoms, with some evidence suggesting that improvements in HRQOL occur in both types of patients[25], and other evidence pointing out that they are only found in symptom-detected patients[10]. Our study also provides some evidence that symptom-detected patients have significantly lower overall scores on the GIQLI and on the EQ preference index than serologic-detected patients, which suggests that the former have better self-perceived health on diagnosis. However, the nature of the present study meant that it was not possible to determine whether diet-induced improvements in HRQOL were related or not to the type of diagnosis.

The only non-disease-related variable that had a significant influence on HRQOL was gender, with women scoring significantly poorer on the GIQLI questionnaire. It has been suggested that in treated CD patients, low scoring of SF-36 is confined to female patients[15]. However, in our study, the influence of gender on HRQOL was less clear because, although female sex was a significant independent variable for poorer perception of HRQOL as measured by the GIQLI, the effect was not seen on either the EQ preference value index or on the EQ VAS.

Our results suggest that untreated newly diagnosed CD has a significant negative impact on several domains of HRQOL, and that HRQOL is more impaired in symptomatic patients and in those with CD-associated diseases. On the other hand, HRQOL improves to levels observed in the general population when CD is controlled with a GFD. From our observations, it can be concluded that the assessment of HRQOL in CD patients is relevant because it improves physicians' knowledge of the implications of the disease, and helps patients to recognize the general impact of the disease. Additionally, the fact that patients following a GFD report similar levels of HRQOL as members of the general population should encourage patients to adhere to the GFD.

## ACKNOWLEDGMENTS

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## COMMENTS

### *Background*

Celiac disease (CD) is a chronic immuno-inflammatory enteropathy that appears in genetically predisposed patients, who develop characteristic small-intestinal-mucosal changes, due to hypersensitivity to gluten. The chronic nature of the condition, together with the limitations imposed by the need to follow a permanent restrictive diet, substantial numbers of physician visits, the risk of associated diseases and potential complications, mean that CD can have a considerable negative impact on health-related quality of life (HRQOL). The impairment of HRQOL in untreated CD and its improvement after treatment with a gluten-free diet (GFD), has been suggested using generic multidimensional HRQOL measures.

### *Research frontiers*

There is a lack of information relative to some areas in the research of HRQOL in CD. There is no agreement as to whether the HRQOL of treated CD patients reaches that of the general population, the factors involved in HRQOL impairment, and in the use of more specific instruments to measure the HRQOL of CD patients.

### *Innovations*

Two instruments to measure HRQOL in two groups of CD patients: 163 patients following a GFD and 177 newly diagnosed patients who were on a normal diet containing gluten. Results on the gastrointestinal quality of life index GIQLI and EuroQol-5D EQ questionnaires suggested that the HRQOL of untreated, recently diagnosed CD patients was significantly impaired, on almost all of the dimensions on both instruments and on the overall score. Patients, before starting a GFD, considered their overall health equivalent to a rating of only fair. CD patients controlled with a GFD reported significantly better HRQOL, and in fact had EQ scores that were very similar to those of a representative sample of the Spanish general population. The importance of diet on CD was confirmed in an ancillary study in which HRQOL was re-measured after at least 6 mo on GFD. Results of that ancillary study confirm that treatment of CD improves HRQOL. Our study also provided some evidence that symptom-detected patients had significantly lower overall scores on the GIQLI and on the EQ preference index than serologic-detected patients, which suggested that the former had better self-perceived health on diagnosis. To determine the factors involved in HRQOL impairment in CD, a multivariate analysis using the condition-specific GIQLI or the generic EQ instrument was performed. Results of that analysis suggested that the variables significantly associated with a worse HRQOL were female gender, failure to follow a GFD, and symptomatic status.

### *Applications*

The results of the present study suggest that compliance with a GFD and adequate control of symptoms are decisive in terms that CD patients may recover to satisfactory levels of self-perceived health. The assessment of HRQOL in CD patients is relevant because it improves physicians' knowledge of the implications of the disease and helps patients to recognize the general impact of the disease. The fact that patients following a GFD reached similar levels of HRQOL as members of the general population should encourage patients to adhere to a GFD.

### Terminology

HRQOL has a recognized importance to evaluate, manage and follow patients with chronic diseases, such as CD. Different types of instruments for measuring HRQOL have been introduced, with the most important being the questionnaires. There are generic questionnaires and disease-specific questionnaires. Although a specific questionnaire for CD has recently been published, the most widely used instruments to measure HRQOL in CD are the generic questionnaires. GIQLI is a self-administered questionnaire designed to assess HRQOL in patients with gastrointestinal diseases. The GIQLI consists of 36 items grouped into five domains of health (gastrointestinal symptoms, physical dysfunction, social dysfunction, emotional dysfunction and treatment effects). Responses are scored on a 4-point Likert scale, in which 4 corresponds to the highest level of functioning. EQ is a self-administered generic utility measure that provides both a descriptive profile and an overall index for HRQOL. The EQ includes five dimensions (mobility, personal care, daily activities, pain and anxiety–depression) answered on a 3-point scale ranging from no problems (level 1) to extreme problems (level 3). Combining one level of severity from each of the five dimensions generates a number of discrete health states that can be assigned preference values ranging from 0, which represents worst health status, to 1 (best health status). The EQ also includes a VAS that ranges from 0 (worst imaginable health status) to 100.

### Peer Review

This is a well designed study addressing an important aspect of celiac disease which adds considerably to what is already known.

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*CLINICAL RESEARCH*

# Clinical value of fecal calprotectin in determining disease activity of ulcerative colitis

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## Abstract

**AIM:** To investigate possibility and clinical application of fecal calprotectin in determining disease activity of ulcerative colitis (UC).

**METHODS:** The enzyme-linked immunosorbent assay (ELISA) was used to measure the concentrations of calprotectin in feces obtained from 66 patients with UC and 20 controls. C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), acid glycoprotein (AGP) were also measured and were compared with calprotectin in determining disease activity of UC. The disease activity of UC was also determined by the Sutherland criteria.

**RESULTS:** The fecal calprotectin concentration in the patients with active UC was significantly higher than that in the inactive UC and in the controls (402.16 ± 48.0 μg/g *vs* 35.93 ± 3.39 μg/g, 11.5 ± 3.42 μg/g, $P < 0.01$). The fecal calprotectin concentration in the inactive UC group was significantly higher than that in the control group ($P < 0.05$). A significant difference was also found in the patients with active UC of mild, moderate and severe degrees. The area under the curve of the receiver operating characteristics ($AUC^{ROC}$) was 0.975, 0.740, 0.692 and 0.737 for fecal calprotectin, CRP, ESR and AGP, respectively. There was a strong correlation between the fecal calprotectin concentration and the endoscopic gradings for UC ($r = 0.866$, $P < 0.001$).

**CONCLUSION:** Calprotectin in the patient's feces can reflect the disease activity of UC and can be used as a rational fecal marker for intestinal inflammation in clinical practice. This kind of marker is relatively precise, simple and noninvasive when compared with other commonly-used markers such as CRP, ESR and AGP.



**Key words:** Fecal calprotectin; Disease activity; Ulcerative colitis; Enzyme-linked immunosorbent assay

## INTRODUCTION

Ulcerative colitis (UC) is a chronic, idiopathic inflammatory bowel disease characterized by remission of disease activity. The incidence and the prevalence rates of UC are increasing in China[1]. It is important to accurately evaluate intestinal mucosa inflammation in the management of these patients, particularly for the assessment of therapeutic effectiveness. Colonoscopy and biopsy are useful in the assessment of intestinal mucosa inflammation of patients with UC, but these examinations can be a heavy burden to the patient[2,3]. Clinical evaluations including laboratory tests such as C-reactive protein (CRP)[4-6], erythrocyte sedimentation rate (ESR)[7], acid glycoprotein (AGP)[8], and platelet count[9,10], have been used for the determination of disease activity of UC, but none of them are specific for gut inflammation[11]. Therefore, a new marker that will be more sensitive and specific for determination of disease activity of UC is urgently needed in clinical practice.

An alternative approach to the assessment of the presence of intestinal inflammation[12] is to analyze the whole gut lavage fluid or to quantitate the protease resistant neutrophil derived proteins such as lactoferrin[13,14] in the patient's feces, and this approach can be non-invasive for the patient. Calprotectin is one of these proteins. It is a major protein in the neutrophilic granulocytes and the macrophages[15], which accounts for 60% of the total protein in the cytosol fraction in these cells[16,17]. This kind of protein can resist metabolic degradation caused by intestinal bacteria, and the protein is relatively stable in stools for up to one week at room temperature[18]. It can differentiate between patients with organic or non-organic intestinal disease, and can be useful in detecting colorectal cancer and inflammatory disorders, and can also be useful in predicting a relapse of inflammatory bowel disease[19].

Our study was aimed at the measurement of the concentration of calprotectin in the feces of the UC patient and at the comparison of it with commonly-used markers in clinical practice, such as CRP, ESR and AGP.

## MATERIALS AND METHODS

### Study subjects

Sixty-six patients with UC (age, 38.97 ± 2.39 years) were enrolled in the study, including 15 patients with proctitis, 22 with left-sided colitis, and 29 with pancolitis. Of the patients, 44 were hospitalized. The patients' disease activities were assessed according to the Sutherland criteria[20] in which a score of more than two was considered to indicate the active stage of the disease. The control group consisted of 20 subjects (age, 38.95 ± 3.59 years) with no confirmed abnormality in the upper or lower digestive tract.

### Methods of stool collection

The patients were instructed to defecate directly into a polystyrene container. The stool samples were stored at -70℃ until the time of measurement.

### Measurement of fecal calprotectin by ELISA

The stool samples were thawed, and 50-100 mg of the sample was suspended with 2500-5000 μL of the fecal extraction buffer, and was homogenized; then, the suspernatant was diluted to 1:50, and the calprotectin was analyzed by the enzyme-linked immunosorbent assay (ELISA) using the Nycotest Phical ELISA kit (Nycomed, Norway). Microcapture and immunoaffinity-purified rabbit anticalprotectin conjugated with alkaline phosphatase was used for the development. The ELISA read the absorbance at 405 nm for 96-well plates. The results of the sample tests were evaluated from the standard curves. CRP, ESR and AGP were measured in the clinical laboratory, West China Hospital, based on the instructions provided by the reagent manufacturer.

### Statistical analysis

Statistical analysis was performed using the statistical package SPSS 11.5. The data were expressed as means ± SD. The Mann-Whitney test was used to assess differences in the laboratory parameters between the groups, and Spearmans' correlation was used to analyze the correlation between the parameters. All the $P$ values were two tailed; $P$ values < 0.05 were considered statistically significant. The receiver operating characteristics (ROC) (sensitivity and specificity) were assessed by the curve analysis as described by Henderson[21].

## RESULTS

### Concentrations of fecal calprotectin, CRP, ESR and AGP in patients with UC and in controls

There was a significant difference in the fecal calprotectin concentration between the patients with active UC and the patients with inactive UC ($P < 0.01$) (Table 1, Figure 1). The calprotectin concentration was significantly greater in the patients with inactive UC than in the controls ($P < 0.05$). The patients with active UC had higher levels of CRP, ESR and AGP than the patients with inactive UC and the controls ($P < 0.05$), but there was no significant difference between the patients with inactive UC and the controls.

### Relationship between the concentrations of fecal calprotectin, CRP, ESR and AGP and the disease activity index (DAI) in UC

As shown in Figure 2, the concentrations of fecal calprotectin, CRP, ESR and AGP in UC had a good correlation with DAI. The correlation coefficients between DAI and the concentrations of fecal calprotectin, CRP, ESR and AGP were 0.866, 0.492, 0.433 and 0.533, respectively. This association was strongest for fecal calprotectin and weakest for ESR.

### Specificity and sensitivity

The correspondence between the DAI-based classification of the active or the inactive disease status and the classification based on the parameter cut-offs was analyzed for each parameter (Table 2) and was expressed as the percentage of the samples that were correspondingly identified (specificity and sensitivity). Specificity was highest for fecal calprotectin, and lowest for AGP. The specificity rates for fecal calprotectin, CRP, ESR and AGP were 79.4%, 69.0%, 68.9% and 65.5%, respectively. The sensitivity for fecal calprotectin was relatively high, but was relatively low for CRP. The sensitivity rates for fecal calprotectin, CRP, ESR and AGP were 91.9%, 62.2%, 64.9%, and 67.6%, respectively. The ROC curves showed the trade-off between specificity and sensitivity for fecal calprotectin (the area under the curve, AUC, 0.975 ± 0.015; $P < 0.001$), for CRP (AUC, 0.740 ± 0.061; $P < 0.001$), for ESR (AUC, 0.692 ± 0.064; $P < 0.01$), and for AGP (AUC, 0.737 ± 0.062; $P < 0.001$) (Figure 3). The $AUC^{ROC}$ of fecal calprotectin was greater than that of CRP, ESR or AGP ($P < 0.01$).

## DISCUSSION

Chronic relapsing and remitting inflammation of the gastrointestinal tract is the hallmark of UC. One of the most prominent histological features observed in UC is infiltration of the neutrophils into the inflamed mucosa at an early stage of inflammation. The neutrophils are major sources of inflammatory cytokines, chemokines proteases, active lipids, and reactive oxygen derivatives, as well as a full complement of factors needed to exacerbate mucosal inflammation and tissue injury[22-25] The fecal calprotectin excretion of indium-labeled autologous granulocytes has for a long time been suggested as the gold standard test in assessing bowel inflammation in inflammatory bowel disease. However, as it involves an exposure to radiation and prolonged fecal collection, which is unpopular with patients and laboratory staff, it is only used as a research tool. In patients with inflammatory bowel disease, a three day excretion of indium-labeled granulocytes correlated well with a daily excretion and a one-off fecal calprotectin level[26].

In this study, we focused on the evaluation of any relationship that might exist between the mucosal neutrophil infiltration represented by calprotectin, CRP, ESR, AGP and the UC disease activity represented by the Sutherland criteria. The DAI score of UC is the sum score of the following four parameters (each scoring

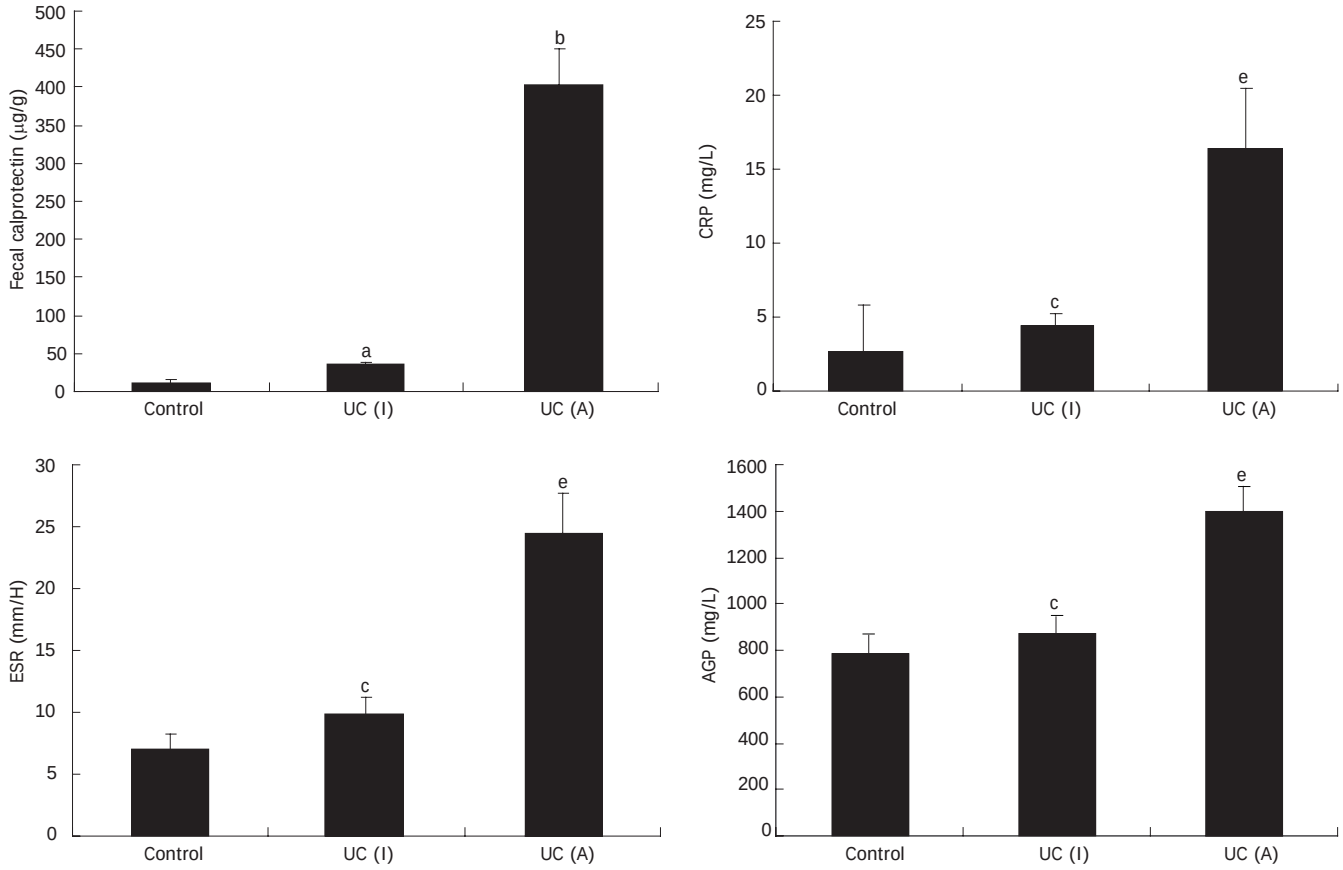


**Figure 1** Concentrations of fecal calprotectin, CRP, ESR and AGP in the UC patients and the controls. UC (A): Ulcerative colitis (active phase); UC (I): Ulcerative colitis (inactive phase). [a]$P < 0.05$ *vs* the control, [b]$P < 0.01$ *vs* UC (I) and the control, [c]$P > 0.05$ *vs* the control, [e]$P < 0.05$ *vs* UC (I) and the control.

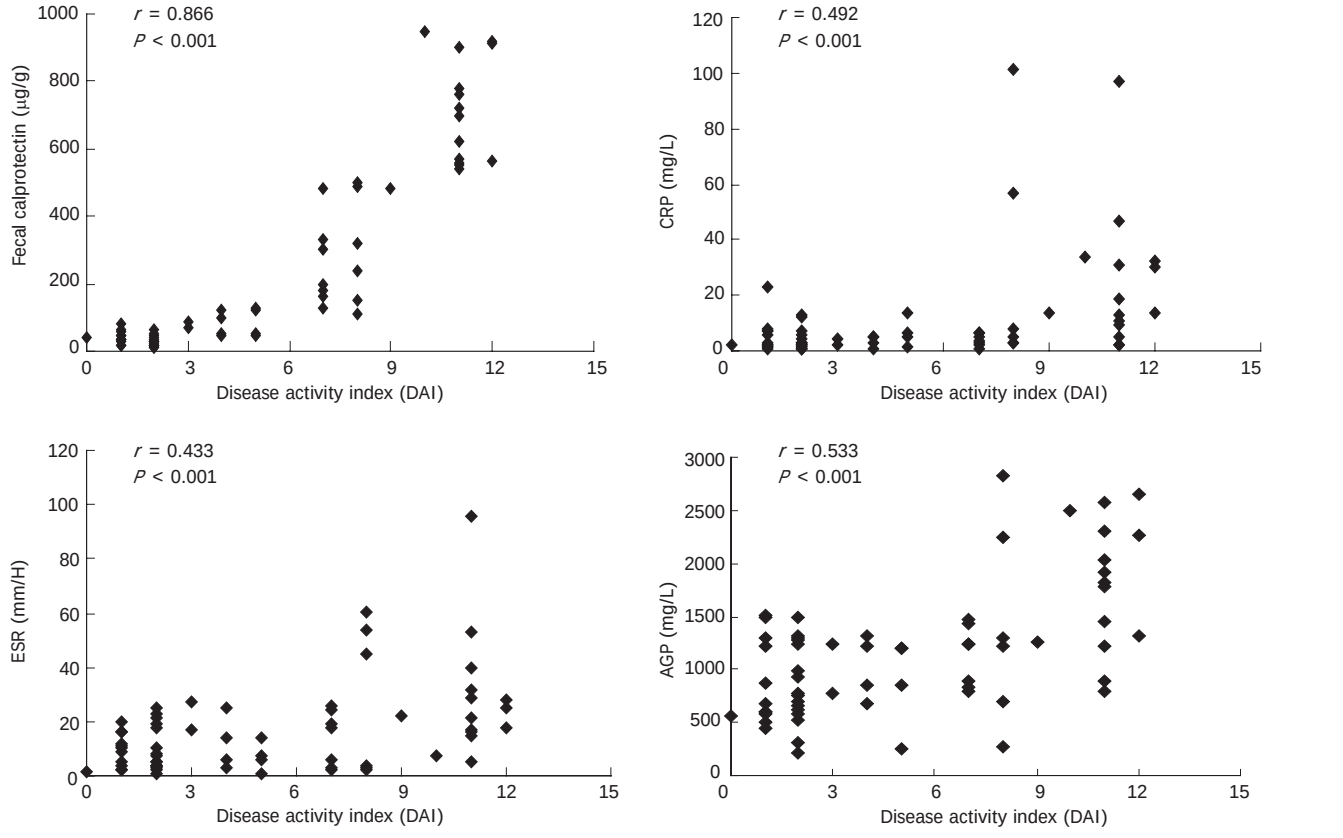


**Figure 2** Concentrations of fecal calprotectin, CRP, ESR and AGP in UC and the DAI score of UC.

**Table 1 Concentrations of fecal calprotectin, CRP, ESR and AGP in the UC patients and the controls (mean ± SD)**

| Group | Calprotectin (μg/g) | CRP (mg/L) | ESR (mm/H) | AGP (mg/L) |
|---|---|---|---|---|
| Control | 11.5 ± 3.42 | 2.66 ± 3.2 | 7.05 ± 1.2 | 786.65 ± 77.65 |
| Inactive UC | 35.93 ± 3.39 | 4.39 ± 0.89 | 9.84 ± 1.36 | 870.14 ± 71.04 |
| Active UC | 402.16 ± 48.0 | 16.45 ± 3.98 | 21.44 ± 3.29 | 1394.9 ± 109.3 |

UC: Ulcerative colitis; CRP: C-reactive protein; ESR: Erythrocyte sedimentation rate; AGP: Acid glycoprotein.

**Table 2 Specificity and sensitivity for fecal calprotectin, CRP, ESR and AGP**

| Marker | Cut-off | Specificity (%) | Sensitivity (%) |
|---|---|---|---|
| Fecal calprotectin | 50.0 μg/g | 79.4 | 91.9 |
| CRP | 5.0 mg/L | 69.0 | 62.2 |
| ESR | 15.0 mm/h | 68.9 | 64.9 |
| AGP | 1200 mg/L | 65.5 | 67.6 |

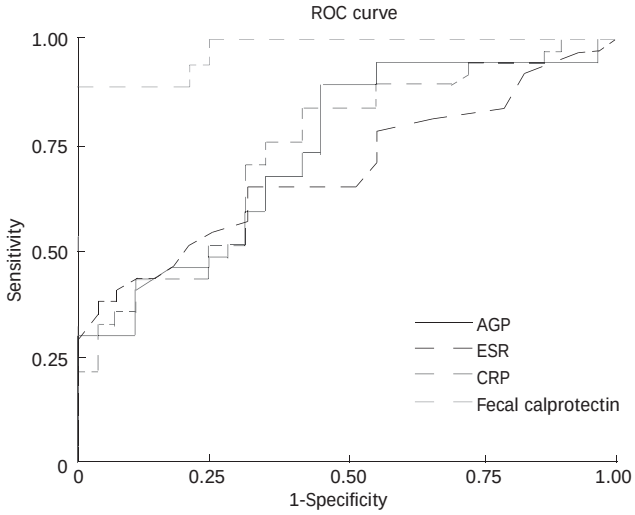


**Figure 3** The ROC curve analysis on the abilities of calprotectin, CRP, ESR and AGP to make a difference between active UC and inactive UC.

between nought and three, making 12 the worst score): stool frequency, rectal bleeding, mucosa appearance, and physician's global assessment[27]. It reflects the clinical representation of UC and is the most extensive and simplest DAI. It is adopted by the guidelines for the management of inflammatory bowel disease in the fourth Asia Pacific Digestive Week[28]. Our results showed that fecal calprotectin concentrations were significantly higher in the patients with active UC than in the patients with inactive UC and in the controls. In addition, our results showed that fecal calprotectin concentration was higher in the patients with inactive UC than in the controls. In the patients with UC, the fecal calprotectin concentration had a better correlation with DAI than the CRP, ESR or AGP concentration.

The ROC curve is adopted not only to ensure the right cut-off point, but also to compare the diagnostic values of two or more diagnostic tests. The ROC analysis on the fecal calprotectin concentrations under these circumstances, showed that a cut-off point of 50.0 μg/g for calprotectin had a 91.9% sensitivity and a 79.4% specificity for making a differentiation between active UC and inactive UC. These were significantly better than those obtained with CRP, ESR and AGP.

In this study, ELISA was used to determine the fecal calprotectin concentrations in the patients with UC and in the controls. In comparison to endoscopy this method is simple, noninvasive and inexpensive. However, fecal calprotectin can only reflect the excretion of neutrophils. Many infective diseases can cause a large number of neutrophils to infiltrate, so that fecal calprotectin is elevated in a number of organic gastroenterological disorders[29-31]. Therefore, fecal calprotectin is not desirable as a method that is required to differentiate efficiently between UC and infective colitis, so it cannot take the place of an endoscope in diagnosing UC. Regardless of how sensitive the calprotectin technique may be in the detection of disease activity in patients with previously diagnosed UC, its greater potential use is in identifying patients with UC and in differentiating between patients with UC and patients with non-inflammatory disorders, still requires further study.

In conclusion, the determination of fecal calprotectin is an objective approach to grading the mucosal disease activity in patients with inflammatory bowel disease. The advantages of fecal calprotectin are simplicity, noninvasiveness, and relatively low cost. Fecal calprotectin can be useful not only in research but also in clinical practice.

## COMMENTS

### *Background*

The incidence and the prevalence rates of ulcerative colitis (UC) are increasing in China. It is important to accurately evaluate intestinal mucosa inflammation in the management of these patients, particularly for the assessment of therapeutic effectiveness. No clinical evaluations are specific for gut inflammation. Therefore, a new marker that will be more sensitive and specific for determination of disease activity of UC is urgently needed in clinical practice.

### *Research frontiers*

Calprotectin can resist metabolic degradation caused by intestinal bacteria, and the protein is relatively stable in stools for up to one week at room temperature. Some foreign research has shown that fecal calprotectin concentrations were significantly higher in patients with active UC than in patients with inactive UC and in the controls.

### *Innovations and breakthroughs*

Our study improved the originality of the manuscript assessing the relationship between calprotectin level and extension of colitis. This question was not described enough in the medical literature.

### *Applications*

The aim of our study was to investigate the possibility and clinical application of fecal calprotectin in determining disease activity of UC. Our findings suggest that calprotectin in the patient's feces can reflect disease activity of UC and can be used as a rational fecal marker for intestinal inflammation in clinical practice.

### *Terminology*

Receiver operating characteristics (ROC) curve is adopted not only to ensure the right cut-off point but also to compare the diagnostic values of two or more diagnostic tests. The accuracy of diagnostic test is characterized by the sensitivity and specificity. A ROC curve displays the sensitivity of a diagnostic test over all possible false-positive rates.

### *Peer review*

This manuscript provided compelling evidence that fecal calprotectin was a better marker than C-reactive protein (CRP), erythrocyte sedimentation rate (ESR) or acid glycoprotein (AGP) and improved the originality of the manuscript assessing the relationship between calprotectin level and extension of colitis. It deserves to be published.

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*RAPID COMMUNICATION*

# Selection of treatment modality for hepatocellular carcinoma according to the modified Japan Integrated Staging score

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## Abstract

**AIM:** To compare the prognosis of patients who underwent hepatectomy and ablation using the modified Japan Integrated Staging score (mJIS).

**METHODS:** We examined the clinicopathologic records and patient outcomes in 278 HCC patients including 226 undergoing hepatectomy and 52 undergoing ablation therapy.

**RESULTS:** Cirrhosis was more frequent in the ablation group. Tumor size, number and presence of vascular invasion were significantly higher in the operation group compared to the ablation group. The local recurrence rate adjacent to treated lesions was significantly higher in the ablation group compared to the operation group ($P < 0.05$). The 3- and 5-year survival rates in the ablation and the operation group were 66% and 78%, and 50% and 63%, respectively, but not significantly different. Over 50% survival rates were observed in patients with a mJIS score of 0-2 in both groups. However, survival rates with a score of 3-5 in both groups were significantly lower.

**CONCLUSION:** According to the mJIS system, both local treatments could be selected for patients with a score of 0-2. However, for patients with a score more than 3, liver transplantation might be a better option in patients with HCC.



**Key words:** Hepatocellular carcinoma; Hepatectomy, Ablation; Modified Japan integrated staging score; Liver transplantation

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## INTRODUCTION

Although hepatic resection is supposed to be the best curative local treatment for hepatocellular carcinoma (HCC)[1-3], sufficient hepatic functional reserve is necessary. In recent years, various treatment modalities have become available for HCC patients and the appropriate treatment should be selected according to tumor staging and liver function[4]. In the past decade, combined staging systems with tumor factors and liver function in HCC patients have been proposed worldwide[4-7]. We also proposed the modified cancer of the liver Italian program (mCLIP) score and the modified Japan Integrated Staging score (mJIS)[8,9]. Comparing the various staging systems using multivariate survival analysis, mJIS is the best available system to predict survival in HCC patients after hepatectomy[10]. The Liver Cancer Study Group of Japan subsequently showed that the mJIS system had good predictive accuracy for survival of Japanese patients with HCC by the records of 42269 patients diagnosed with HCC registered between 1992 and 1999 in a nationwide Japanese database[11].

Liver transplantation (LT) has recently been considered a good option to cure some HCC patients with poor hepatic function, such as Child-Pugh C cirrhosis[12]. The usefulness of cadavaric liver transplantation (LT) for HCC treatment has been clarified in Western countries[13] and, furthermore,

Todo *et al* reported good results in HCC treatment with the living related LT in Japan[14]. Therefore, LT seems to be a better treatment option in some patients who undergo local treatments in Japan. At this stage, Milan or University of California, San Francisco (UCSF) criteria using tumor factors and the Barcelona Clinic Liver Cancer (BCLC) system using tumor, liver function and performance status, have been used to decide the indication of LT[4,15,16]. It has not yet been clarified whether the mJIS system could be useful in the selection of LT in HCC patients.

In this study, we compare patient demographics, preoperative liver function, tumor parameters and long-term patient survival prognosis of 278 HCC patients who underwent hepatectomy and ablation using mJIS at several cancer institutions in Nagasaki prefecture, Japan. We then discuss the selection of treatment by comparing results by LT. Our aim is to clarify the treatment selection criteria for HCC patients using mJIS.

**Table 1A Definition and criteria of the TNM stage for HCC according to the Liver Cancer Study Group of Japan[17]**

| Factor of T category | |
|---|---|
| 1 Number of tumors: 1 | |
| 2 Tumor size: no more than 2 cm | |
| 3 No vascular or bile duct invasion | |
| T category | T1: Fulfilling all three factors |
| | T2: Fulfilling two factors |
| | T3: Fulfilling one factor |
| | T4: Fulfilling none of the factors |
| N category | N0: Absence of lymph node metastasis |
| | N1: Presence of lymph node metastasis |
| M category | M0: Absence of distant metastasis |
| | M1: Presence of distant metastasis |
| Stage Ⅰ | T1 N0 M0 |
| Stage Ⅱ | T2 N0 M0 |
| Stage Ⅲ | T3 N0 M0 |
| Stage Ⅳ-A | T4 N0 M0 or T1-T4, N1M0 |
| Stage Ⅳ-B | T1-4, N0 or 1, M1 |

## MATERIALS AND METHODS

### Patients

We analyzed 278 patients with HCC who underwent surgical resection or ablation treatments in the Division of Surgical Oncology and the First and Second Department of Internal Medicine, Nagasaki University Graduate School of Biomedical Sciences (NUGSBS), and its related hospitals between 1994 and 2005. The study design was approved by the Human Ethics Review Board of our institution. Informed consent for data collection was obtained from each patient during this period. Anesthetic and patient data were retrieved from the NUGSBS database. Tumor stage and curability were determined according to the *Classification of Primary Liver Cancer*[17]. Subjects were divided into two groups: (1) Operation group with 226 patients. Preoperative treatment was performed in 78 patients including chemoembolization in 69 and thermal ablation in nine. Operative procedures included hemihepatectomy in 56 patients, anatomical sectriectomy in 74 and partial resection in 96. (2) Ablation group with 52 patients, including alcohol injection in 15 patients, radio-frequency ablation (RFA) in 32, and microwave coagulation therapy (MCT) in five patients.

### Treatment indications, procedures and follow-up

The volume of liver to be resected was estimated according to results of the indocyanine green retention rate at 15 min (ICG R15) using Takasaki's formula[18]. Furthermore, hepatic function for hepatectomy was limited as ICGR15 < 40%, Child-Pugh classification A or B, and total bilirubin level < 2 mg/dL according to Miyagawa's criteria[19]. The expected liver volume for resection, excluding the tumor ($cm^3$), was measured by computed tomography (CT) volumetry[20]. Radical hepatectomy was performed to remove the hepatic tumor without leaving any residual tumor. The indications for hepatic resection of the size and the number of HCC were more than 2 cm, and less than or equal to three lesions, respectively. Distant metastasis was an extra-indication for hepatectomy. The assessment of tumor factors in the operation group was confirmed by histopathological examination of the resected specimen. We used the histopathological factors and curability by hepatectomy of the *Liver Cancer Study Group of Japan* by the *Classification of Primary Liver Cancer*[17].

For RFA or MCT, the indication for hepatic resection of the size and the number of HCC were less than 3 cm, and less than or equal to three lesions, respectively[21]. Hepatic function for ablations is limited as Child-Pugh classification A or B, platelet counts more than 50 000/$mm^3$, prothrombin activity more than 50% and total bilirubin level less than 3 mg/dL. When the appropriate coagulation was estimated to be incomplete by percutaneous puncture, ablations under laparoscopy, thoracoscopy or laparotomy were selected[22,23]. Evaluation of vascular involvement was performed by image analysis, such as enhanced computed tomography or magnetic resonance imaging.

After discharge from hospital, the patient status, laboratory data, and disease recurrence were checked every two to three months.

### Staging criteria of the modified Japan Integrated Staging score (mJIS)

The assessment of each factor was confirmed by histopathological examination of the resected specimen, or by computed tomography scan, ultrasonography, magnetic resonance image or angiography. We used the pathological tumor-node-metastasis (pTNM) classification system of the Liver Cancer Study Group (LCSG) of Japan in 2000[17]. T category is determined by three factors of number, size and vascular or bile duct invasion. N category is the presence of lymph node metastasis and M category is the presence of distant metastasis. TNM staging has four stages according to T, N and M categories (Table 1A). Classification of Child-Pugh[24] and liver damage grade by LCSG[17] are shown in Table 1B. The original JIS score proposed by Kudo *et al* comprised the sum of points for two variables of the Japanese TNM classification and Child-Pugh classification[6]. In the modified JIS score proposed by our institute[9,10], the Child-Pugh classification score was replaced by that of liver damage grade by the LCSG of Japan (Table 1C).

**Table 1B Definition and criteria of Child-Pugh classification and liver damage grade**

| | | | |
|---|---|---|---|
| Child-Pugh classification[24] | A | B | C |
| Encephalopathy | none | mild | coma |
| Ascites | none | responsive | unresponsive |
| Serum bilirubin (mg/dL) | < 2.0 | 2.0-3.0 | > 3.0 |
| Serum albumin (g/dL) | > 3.5 | 2.8-3.5 | < 2.8 |
| Prothrombin activity (%) | > 70 | 40-70 | < 40 |
| Liver damage grade[17] | A | B | C |
| Ascites | none | responsive | unresponsive |
| Serum bilirubin (mg/dL) | < 2.0 | 2.0-3.0 | > 3.0 |
| Serum albumin (g/dL) | > 3.5 | 3.0-3.5 | < 3.0 |
| ICG R15 (%) | < 15 | 15-40 | > 40 |
| Prothrombin activity (%) | > 80 | 50-80 | < 50 |

**Table 1C Definition and criteria of the JIS and the mJIS**

| | Score | | | |
|---|---|---|---|---|
| | 0 | 1 | 2 | 3 |
| Original JIS score[6] | | | | |
| Japanese TNM stage | I | II | III | IV |
| Child-Pugh Classification | A | B | C | |
| Modified JIS score[9] | | | | |
| Japanese TNM stage | I | II | III | IV |
| Liver damage grade | A | B | C | |

TNM: Tumor-node metastasis.

### *Statistical analysis*

Continuous data were expressed as the mean ± SD. Data from different groups were compared using one-way analysis of variance (ANOVA) and examined by student's *t*-test or Dunnet's multiple comparison test. For univariate analysis, categorical data were analyzed by the Fisher's exact test. Disease-free and overall survival rates were calculated according to the Kaplan-Meier method, and differences between groups were tested for significance using the log-rank test. Multivariate analysis was performed using the proportional hazards regression model. A two-tailed *P* value of < 0.05 was considered significant. Statistical analyses were performed using SAS software (Statistical Analysis System Inc., Cary, NC).

## RESULTS

Patient age, gender and period of treatment were not significantly different between groups (Table 2). Rates of cirrhosis and Child-Pugh B were significantly higher in the ablation group. Thirty-five percent of patients underwent pretreatment in the operation group; however, no patients underwent other treatments in the ablation group. Tumor size in the operation group was significantly larger than that in the ablation group. The number of tumors and rate of vascular involvement were significantly higher in the operation group compared to the ablation group. Posttreatment adjuvant treatments were similarly performed in both groups.

In the ablation group, tumor relapse was observed in 22 patients (42%) including 10 with intrahepatic metastasis and 12 with local recurrence adjacent to the ablated site (Figure 1). On the other hand, in the operation group, tumor relapse was observed in 122 patients (62%), which included 117 with intrahepatic metastasis and five with local recurrence adjacent to the resected margin. The local recurrence rate adjacent to the treated lesion was significantly higher in the ablation group compared to the operation group ($P < 0.05$).

By applying mJIS, discrimination of survival in each score was remarkable (Figure 2). The 3- and 5-year survival rates in the ablation and operation group were 66% and 78%, and 50% and 63%, respectively (Figure 3); however, there were no significant differences between groups. Child-Pugh B was significantly associated with poor disease-free and overall survival (Table 3). Multiple tumors were associated with overall survival. However, difference of treatment modality was not associated with prognosis in the present study. Table 4 shows 3-year survival rates in each score of mJIS; however, there were no significant

**Table 2 Patient demographics between two groups in HCC patients**

| | Operation (*n* = 226) | Ablation (*n* = 52) | *P*-value |
|---|---|---|---|
| Age (yr) | 60.2 ± 10.5 | 58.3 ± 10.7 | 0.074 |
| Gender | | | |
| male/female | 179/43 | 37/15 | 0.283 |
| Time to treatment (yr)[1] | (5.1, 8.4, 11.2) | (5.4, 9.3, 11.6) | 0.28 |
| Background liver | | | |
| chronic hepatitis/cirrhosis/normal | 119/94/13 | 4/48/0 | < 0.001 |
| Hepatitis virus | | | |
| B/C/B&C/non-B non-C | 72/116/11/27 | 11/36/5/0 | 0.007 |
| Child-Pugh classification | | | |
| A/B | 201/25 | 34/18 | < 0.001 |
| Pretreatment | | | |
| Yes/No | 78/148 | 0/52 | < 0.001 |
| Tumor size | | | |
| < 5 cm/ ⩾ 5 cm | 160/66 | 49/3 | < 0.001 |
| Number of tumors | | | |
| solitary/multiple | 174/52 | 37/15 | 0.479 |
| Vascular involvement | | | |
| No/Yes | 162/64 | 48/4 | 0.003 |
| Adjuvant therapy | | | |
| Yes/No | 5/221 | 0/52 | 0.615 |

[1]Each triplet gives the 25th, 50th and 75th sample percentiles. Time to the treatment since 1 January 1994.

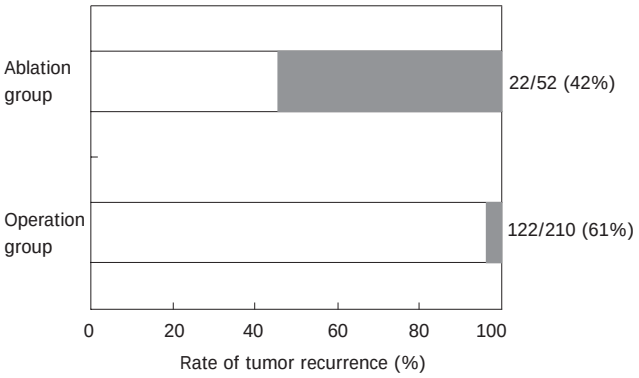


**Figure 1** Tumor relapse and site of recurrence after treatment in two groups. Open square shows intrahepatic metastasis and closed square shows the local recurrence adjacent to treated lesion.

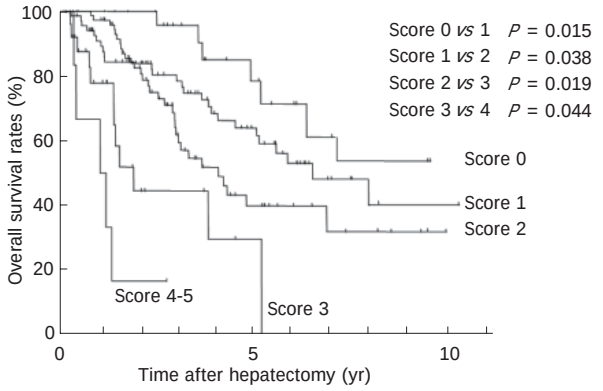


**Figure 2** Survival using mJIS in HCC patients who underwent hepatic resection.

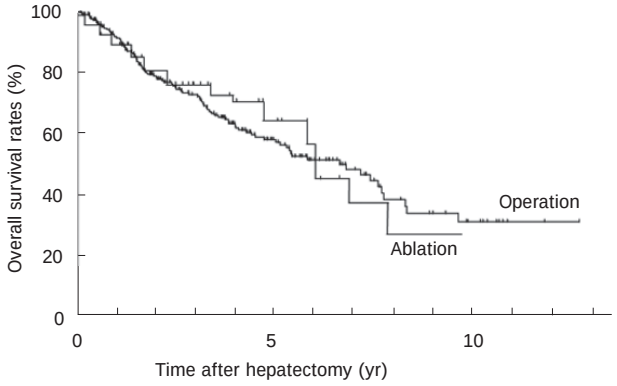


**Figure 3** Comparison of survival in HCC patients between operation and ablation groups.

**Table 3 Survival between two groups in HCC patients by multivariate analysis**

| | Disease-free survival | | Overall survival | |
|---|---|---|---|---|
| | HR (95% CI) | *P*-value | HR (95% CI) | *P*-value |
| Child-Pugh classification | | | | |
| B *vs* A | 2.05 (1.25-3.35) | 0.004 | 2.46 (1.38-4.41) | 0.002 |
| Tumor size | | | | |
| ⩾ 5 *vs* < 5 cm | 1.26 (0.83-1.92) | 0.282 | 1.30 (0.75-2.25) | 0.350 |
| Macroscopic findings[1] | | | | |
| Confluent type *vs* Simple nodular type | 1.10 (0.75-1.61) | 0.626 | 1.58 (0.92-2.72) | 0.098 |
| Number of tumor | | | | |
| Multiple *vs* Solitary | 1.23 (0.82-1.85) | 0.306 | 1.73 (1.03-2.91) | 0.037 |
| Vascular involvement | | | | |
| Yes *vs* No | 1.24 (0.68-2.27) | 0.481 | 1.76 (0.93-3.31) | 0.080 |
| Alpha-feto protein level | | | | |
| ⩾ 400 *vs* < 400 ng/mL | 1.41 (0.93-2.11) | 0.103 | 1.36 (0.81-2.28) | 0.250 |
| Treatment modality | | | | |
| Ablation *vs* Hepatectomy | 0.75 (0.46-1.22) | 0.239 | 0.64 (0.39-1.34) | 0.334 |

[1]Macroscopic findings defined by the *Classification of Primary Liver Cancer*[17].

**Table 4 The 3-year survival rates in each score of mJIS after treatments in HCC patients**

| | Operation group (*n* = 226) (%) | Ablation group (*n* = 52) (%) |
|---|---|---|
| mJIS 0 | 96 | 98 |
| mJIS 1 | 80 | 73 |
| mJIS 2 | 66 | 70 |
| mJIS 3 | 39 | 48 |
| mJIS 4 | 35 | 28 |
| mJIS 5 | 25 | - |

differences between the two groups. Overall survival rates between score 2 and 3 were remarkably different in both groups. Over 50% survival rates were obtained up to score 2 in both groups; however, survival rates over score 3 in both groups were lower.

## DISCUSSION

With recent advances in the ablation technique, local tumor control has improved[25]. In comparison with alcohol injection, the modality option or patient survivals have been remarkably improved in the era of RFA or MCT[25-28]. Alcohol injection is not recommended at present[29,30]. In the present study, the ablation group included patients undergoing alcohol injection; however, local control was relatively good because of the small HCC in our series. At this stage, we mainly performed RFA regardless of tumor size, number and location and we also applied RFA under laparotomy or laparoscopy to achieve complete ablation. Selection bias for treatment was shown by our results. Hepatectomy was mainly selected in patients with chronic hepatitis or Child-Pugh A, while ablation was used in patients with impaired liver function such as cirrhosis or Child-Pugh B. In the latter, surgical resection is usually avoided. Concerning tumor factors, hepatectomy was preferably selected for tumors of larger size, and solitary and vascular involvement by the image examinations. Ablation tended to be selected for small and multiple tumors. Wakai *et al* also showed a similar tendency to select the treatment modality[31]. However, Shiina *et al* described the superiority of RFA compared to hepatectomy[32]. Therefore, superiority or selection criteria between both treatments are still controversial. In the recent Japanese guidelines for HCC, the indication of these treatments was not clearly discriminated[33]. In the operation group, other treatments were preferably performed in one-third of all patients. In these patients, ablation therapy was included as well. Based on these results, the background in both groups was remarkably different, which was also the case in Wakai's report[31].

The pattern of tumor relapse was different in the present study and local recurrence adjacent to the ablated section was significantly higher regardless of careful ablations with a sufficient ablation margin more than 5 mm[34]. Some investigators reported that the complete ablation rate is around 90% with HCC and less than 5 cm could be treated[27-29,35]. However, local recurrence in patients undergoing thermal ablation therapy ranged between 9.2% and 13.6%[26-28]. Hong *et al* reported that the local recurrence rate in ablation therapy was higher than that in hepatic resection[36]. Therefore, local control by hepatectomy is superior to that by thermal ablation at this stage, based on the above reports[26-28,31,35] and our results. Although the rate of distant liver metastasis might not be remarkably different based on previous reports[26-28,35,36], tumor recurrence in the distant liver was still high in the operation group in the present study, which might be

associated with the advanced stage of HCC as shown in the results.

With respect to patient survival after treatment, superiority between both groups was not clarified, in addition to survival rate, in our results. We applied the mJIS system in this study, which is the best available to predict HCC patient survival after curable treatments[9-11]. By applying this system, survival in each score in the present series was well discriminated. At this stage, indication of treatment modality in HCC patients with early tumor stage and Child-Pugh A or B between hepatectomy and thermal ablation has been controversial[31,32,34,37]. In patients with small HCC less than 2 cm or in patients with impaired liver function such as a Child-Pugh B, survival benefit was similar between both groups[31,34,37,38]. As described above, local recurrence rate by thermal ablation was higher compared to that by hepatectomy; however, overall survival was not significantly different by previous reports[26-28,34-37]. The guideline for diagnosis and treatment in HCC patients was first proposed by Makuuchi *et al*[33]; however, ablation and hepatectomy were at similar locations in HCC patients with less than four sites and good liver function. Our results showed that the overall survival was similar between both groups, which also had similar mJIS scores, although the included tumor factor and liver function were different between groups, as shown in patient demographics. By multivariate analysis, the difference of treatment modality was not observed in the present study. Up to mJIS score 2, the 3-year survival rate was well maintained; however, survival rate over mJIS score 3 was significantly decreased in both groups. We considered that this border between a score of 2 and 3 might be important to decide the limitation of both conventional treatments. The ultimate curable option should be a liver transplantation. Todo *et al* reported posttransplant survival in HCC patients who met Milan criteria undergoing living-related liver transplantation in Japan[14]. The 3-year survival rates with or without Milan criteria was 79% and 60%, respectively. This report was a satisfactory result at this stage. Compared to this result, even in a group with Milan criteria not met, survival rates over score 3 in both groups were lower than those in HCC patients undergoing living-related LT. In HCC patients with mJIS score 3-5, patients without remarkable vascular involvement would be included in the indication of liver transplantation. By comparing the survival benefit, transplantation is strongly recommended to improve patient prognosis. In patients with score 0-2, the definition of treatment criteria between groups seemed to be difficult on the present evidence and consensus[39,40]. A study with a large series in Japan will clarify this problem in the near future. Our results did not show superiority or definite indication between both treatment modalities in the present study. To improve the survival results in HCC patients, a combination of both treatments[41], chemoembolization[42], or intra-operative ablation under laparoscopy or laparotomy should be used[22,43].

In conclusion, hepatectomy tended to be selected in patients with better functional liver reserve and, hence, ablation therapy tended to be selected in patients with poor hepatic function in our series. In the ablation group, local recurrence near the treatment region tended to be more than that in the hepatectomy group. By multivariate analysis, macroscopic finding and vascular invasion were significant risk factors, but treatment modality was not a prognostic factor. According to the mJIS system, both treatments could be selected for patients with score 0-2; however, for patients with a score more than 3, liver transplantation might be a better option compared to conventional local treatments.

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*RAPID COMMUNICATION*

# Role of 18F-fluorodeoxyglucose positron emission tomography imaging in surgery for pancreatic cancer

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## Abstract

**AIM:** To evaluate the role of positron emission tomography using 18F-fluorodeoxyglucose (FDG-PET) in the surgical management of patients with pancreatic cancer, including the diagnosis, staging, and selection of patients for the subsequent surgical treatment.

**METHODS:** This study involved 53 patients with proven primary pancreatic cancer. The sensitivity of diagnosing the primary cancer was examined for FDG-PET, CT, cytological examination of the bile or pancreatic juice, and the serum levels of carcinoembrionic antigens (CEA) and carbohydrate antigen 19-9 (CA19-9). Next, the accuracy of staging was compared between FDG-PET and CT. Finally, FDG-PET was analyzed semiquantitatively using the standard uptake value (SUV). The impact of the SUV on patient management was evaluated by examining the correlations between the SUV and the histological findings of cancer.

**RESULTS:** The sensitivity of FDG-PET, CT, cytological examination of the bile or pancreatic juice, and the serum levels of CEA and CA19-9 were 92.5%, 88.7%, 46.4%, 37.7% and 69.8%, respectively. In staging, FDG-PET was superior to CT only in diagnosing distant disease (bone metastasis). For local staging, the sensitivity of CT was better than that of FDG-PET. The SUV did not correlate with the pTNM stage, grades, invasions to the vessels and nerve, or with the size of the tumor. However, there was a statistically significant difference (4.6 ± 2.9 *vs* 7.8 ± 4.5, $P = 0.024$) in the SUV between patients with respectable and unresectable disease.

**CONCLUSION:** FDG-PET is thus considered to be useful in the diagnosis of pancreatic cancer. However, regarding the staging of the disease, FDG-PET is not considered to be a sufficiently accurate diagnostic modality. Although the SUV does not correlate with the patho-histological prognostic factors, it may be useful in selecting patients who should undergo subsequent surgical treatment.



**Key words:** Pancreatic cancer; Fluorodeoxyglucose positron emission tomography; Computed tomography; Standard uptake value; Carcinoembrionic antigens; Carbohydrate antigen 19-9; Prognostic factor

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## INTRODUCTION

Today, despite advances in diagnosing modalities, most patients with pancreatic cancer are still unresectable at the time of diagnosis. Surgery remains the only potential for long-term survival, with a resectability rate of around 15%-20% in the latest review[1]. Even in patients with resectable disease, the 5-year survival rate is still around 20%[1-3]. For patients with unresectable disease, chemotherapy with or without radiation therapy is usually chosen, and the survival benefit of chemotherapy with Gemcitabine over 5-fluorouracil has recently been reported[4]. Clearly, an effort has to be made for diagnosing early stage cancer, also by determining its clinical stage and by accurately predicting the prognosis, thus avoiding unnecessary surgical explorations.

Recently, the advantages of positron emission tomography using 18F-fluorodeoxyglucose (FDG-PET) on diagnosing pancreatic cancer, especially small lesions less than 2 cm in size, over the conventional modalities, including computed tomography (CT), have been reported[2]. Furthermore, FDG-PET has also been reported to possibly play a role in predicting the prognosis of pancreatic cancer[2,5,6]. However, the significance of FDG-PET imaging in the management of pancreatic cancer, including diagnosis, staging, or

predicting prognosis, has not yet been established. In this study, we explored the role of FDG-PET in surgery for pancreatic cancer by examining its sensitivity in diagnosing and staging, and its potential for predicting prognosis by comparing the standard uptake value (SUV) and the histological findings.

## MATERIALS AND METHODS

### Patients

This study involved 53 patients with histologically (30 patients) or clinically (23 patients) proven primary pancreatic cancer who had undergone FDG-PET from January 2004 to January 2007. In clinically proven pancreatic cancer patients metastatic lesions were detected in the liver, lymph nodes, or the other organs, by the following up CT retrospectively, and the patients died of the primary pancreatic cancer. The patients' mean age was 70.1 years (range from 44 to 84 years). The patients consisted of 33 males and 20 females. The localisation of the cancer was in the head of the pancreas in 32 patients and in the body and tail in 21 patients. Twenty-eight patients presented with diabetes requiring insulin therapy. Among the 53 patients, 28 patients were diagnosed to be unresectable, and 25 patients eventually underwent surgery with a curative intention, although in 7 of them the cancer turned out to be unresectable because of the intraoperative findings.

### Methods

The sensitivity of diagnosing pancreatic cancer was examined for FDG-PET, CT, cytological examination of the bile or pancreatic juice, and the serum levels of carcinoembrionic antigens (CEA) and carbohydrate antigen 19-9 (CA19-9). Next, the accuracy of staging of the disease and the impact on patient management was evaluated and compared between FDG-PET and CT. Finally, FDG-PET was analyzed semiquantitatively using the standard uptake value (SUV). In the patients who underwent surgery, a correlation was found between the preoperatively obtained SUV and the histological findings of the specimen in regard to the pathological stagings of cancer classified based on the UICC TNM classification (6$^{th}$ edition, 2002), histological differentiation, lymphatic invasions, vascular invasion, and intrapancreatic nerve invasion. The SUV was compared between the resectable and unresectable disease patient groups. A correlation between the SUV and the maximum diameter of the primary lesion, which was determined on CT, was examined. All of the studies were performed retrospectively by collecting and analyzing data from the patient records.

### Cytological examination of the bile or pancreatic juice

The bile or pancreatic juice, which was collected from 28 patients after brushing endoscopic retrograde cholangiopancreatography (ERCP), was subjected to a cytological examination. This was performed using the Papanicolaou staining, and the diagnosis established according to the Papanicolaou classification. The results in Classes Ⅰ, Ⅱ, or Ⅲ were counted as negative in this study; the results in Classes Ⅳ or Ⅴ were counted as positive.

### CT

For multidetector CT scans, a contrast enhancement was performed for each patient. Helical images of the abdomen were routinely obtained and reconstructed with 5 mm thickness. The CT images were interpreted independently and consecutively by two radiologists with extensive experience of more than 10 years in CT scanning. The findings of the CT scans were considered positive when both of the radiologists strongly suspected malignant disease due to a discrete low-attenuation mass within the pancreas, and/or involving the adjacent vessels, and/or swelling of the regional lymph nodes.

### FDG-PET

The FDG-PET images were acquired with PET machine (Siemens EXACT HR+, CTI, Knoxville, TN, USA). The patients were required to fast for at least 4 h before PET imaging. One hour after the intravenous administration of 5 mCi of FDG, the emission images were acquired. The transmission images were acquired to correct for attenuation. The FDG-PET images were interpreted independently and consecutively by two radiologists with extensive experience in FDG-PET imaging. The findings were considered to be positive when both of the radiologists strongly suspected malignant disease. In addition, the images were analyzed semiquantitatively using the SUV, as reported elsewhere[7]. Briefly, regions of interest measuring 1.0 cm$^2$ were drawn over the area of maximum activity in a lesion. The SUV was calculated as follows:

SUV = (activity in region of interest in mCi)/(injected dose in mCi/weight in kg).

### Statistical analysis

The chi-square test was employed for a statistical comparison of the sensitivity of FDG-PET and CT. The Student's *t* test was used to compare the values of the SUV between the two groups. Finally, correlations between the SUV and the maximum diameter of the primary lesion determined on CT were examined by the Pearson's correlation test. All statistical analyses were performed using the SPSS software program (SPSS, Chicago, USA). A $P$ value $< 0.05$ was considered statistically significant.

## RESULTS

### Sensitivity for diagnosing pancreatic cancer

The sensitivity of FDG-PET, CT, cytological examination of the bile or pancreatic juice, and the serum levels of CEA and CA19-9 were 92.5% (49/53), 88.7% (47/53), 46.4% (13/28), 37.7% (20/53) and 69.8% (37/53), respectively.

### Sensitivity for preoperative staging

Among the 53 patients, 28 patients were diagnosed to be unresectable based on the following preoperative imaging findings: invasion to major arteries such as the supramesenteric artery, celiac trunks, or common hepatic artery in 9 patients, para-aortic lymph node metastasis in 12 patients, detection of hepatic metastasis in 14 patients,

**Table 1 Histological findings of the patients**

| Histological findings | | | Number of patients | SUV (mean ± SD) | Difference |
|---|---|---|---|---|---|
| Pathological stage (TNM) | | | | | |
| pT | pN | stage | | | |
| 1 | 0 | ⅠA | 1 | 3.59 | |
| 1 | 1 | ⅠB | 1 | 3.14 | |
| 3 | 0 | ⅡA | 8 | 4.1 ± 0.9 | NS |
| 1, 2, 3 | 1 | ⅡB | 8 | 5.9 ± 4.5 | |
| Histological differentiation | | | | | |
| Well | | | 7 | 3.7 ± 0.7 | NS |
| Moderately-poorly | | | 11 | 5.6 ± 3.6 | |
| Lymphatic invasions | | | | | |
| Negative | | | 9 | 5.3 ± 3.9 | NS |
| Positive | | | 9 | 4.5 ± 2.1 | |
| Venous invasions | | | | | |
| Negative | | | 6 | 6.2 ± 5.1 | NS |
| Positive | | | 12 | 4.4 ± 1.9 | |
| Intrapancreatic nerve invasions | | | | | |
| Negative | | | 11 | 4.8 ± 3.5 | NS |
| Positive | | | 7 | 5.1 ± 2.3 | |

SUV: Standard uptake value; NS: Not significant.

detection of bone metastasis in 7 patients, strongly suspected peritoneal dissemination in 5 patients, general complications associated with aging in 4 patients. Among the 25 patients who eventually underwent surgery with a curative intention, the cancer turned out to be unresectable in 7 of them because of the following intraoperative findings: detection of hepatic metastasis (5 to 8 mm in diameter) in 5 patients, histologically confirmed metastases in the para-aortic lymph node (less than 1 cm in diameter) in 2 patients, and cytologically positive ascites in 2 patients. Therefore, the staging diagnostic sensitivity of FDG-PET and CT was 2/9 (22.2%) and 9/9 (100%) for invasion into the major arteries, 8/14 (57.1%) and 11/14 (78.6%) for para-aortic regional lymph nodes metastases, 10/19 (52.6%) and 14/19 (73.7%) for hepatic metastases, 8/8 (100%) and 1/8 (12.5%) for bone metastases, 3/7 (42.9%) and 4/7 (57.1%) for peritoneal dissemination, respectively. There was a statistically significant difference in the sensitivity detecting invasion into the major arteries ($P = 0.001$) and bone metastases ($P = 0.001$), based on the chi-square test.

### *Correlation between the SUV and histological findings*

In 18 patients, a surgical resection with a lymphadenectomy was performed. A pancreaticoduodenectomy was performed in 11 patients, while a distal pancreatectomy was performed in 7 patients. In these patients, a pathohistological examination was performed. As Table 1 shows, no statistically significant difference was found in any of the histological findings listed in Table 1.

### *Comparison of SUV between resectable and unresectable disease*

As Figure 1 shows, the SUV of the primary lesion was 4.6 ± 2.9 (mean ± SD) in the resectable disease cases, while it was 7.8 ± 4.5 in the unresectable cases, with a statistically significant difference ($P = 0.024$) by the Student's *t*-test. On the other hand, the maximum diameter of the primary lesion measured on CT was 27.9 ± 10.9 mm in the resectable cases, and 40.5 ± 21.4 mm in the unresectable ones ($P = 0.053$). Furthermore, we found no correlation between the SUV and the maximum diameter of the primary lesion measured on CT by using Pearson's correlation test ($P = 0.274$), as shown in Figure 2.

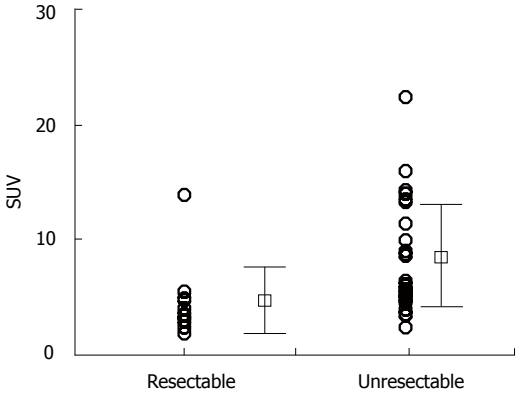


**Figure 1** Comparison of the SUV of the primary lesion between patients with resectable and unresectable disease. SUV: Standard uptake value.

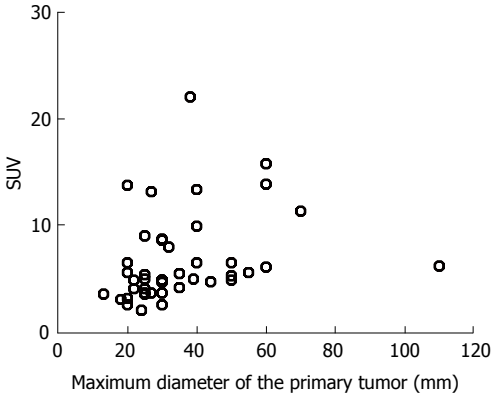


**Figure 2** Relationship between the SUV and the maximum diameter of the primary lesion.

## DISCUSSION

Pancreatic cancer is one of the most lethal human cancers and it continues to be a major unsolved health problem worldwide. Despite efforts in the past 50 years, conventional treatment approaches, such as surgery, radiation, chemotherapy, or combination of these, have had little impact on the course of this aggressive neoplasm[1]. Although recent progress in systemic chemotherapy has been reported[8], surgery remains the only hope for long-term survival[9]. In surgery for pancreatic cancer, a great deal of effort has been made to expand the resection with an extended lymphadenectomy in order to improve the outcome[10]. However, only patients with localized disease and a tumor size less than 2 cm with no lymph node metastases can expect long-term survival after surgery[1,2,11]. Therefore, increased efforts should be focusing not only on diagnosing the early stage disease, but also on staging and predicting prognosis so that unnecessary surgical exploration may be avoided.

Recently, PET has been reported to have superiority to CT, US, and EUS in its sensitivity and specificity in diagnosing pancreatic cancer[2,12]. Furthermore, regarding the role of PET in diagnosing the disease, the metabolic activity of the tumor may be of prognostic significance[5,6]. However, the role of PET in the management of pancreatic cancer has yet to be established.

### *The role of FDG-PET in diagnosis of primary cancer*

The sensitivity of FDG-PET was 92.5%, and it was better than that of CT, cytological examinations of the bile or pancreatic juice, and the serum levels of CEA and CA19-9. Our findings also correlate with those of previous reports, in which the sensitivity of FDG-PET, CT, and US has been reported to be 94%, 89%, and 89%, respectively[12]. On the other hand, in another study, the sensitivity of FDG-PET was found to be lower than or equal to CT[13-15] and these findings thus remain controversial. In particular, when a multidetector CT is performed routinely with thin sections (1 mm), the sensitivity of the CT may further improve[2]. This study was conducted among patients with the histologically or clinically proven pancreatic cancer, and therefore the specificity was not evaluated.

### *The role of FDG-PET in staging of the disease*

This study showed that, as far as staging is considered, FDG-PET was superior to CT only in diagnosing distant disease. On the other hand for local staging the sensitivity of CT was better than that of FDG-PET. As described[2], the poor spatial resolution of FDG-PET limits the local staging of pancreatic cancer. Therefore, the anatomical imaging modalities with CT are better suited to demonstrate the relationship of the tumor, adjacent organs, and vascular structure.

In the nodal staging of the disease, there was no difference between FDG-PET and CT, since both performed poorly. The reported sensitivities of FDG-PET have varied between 46% and 71%[2,16]; thus the results of this study are considered to correlate with these previous reports. It has been said that one possible reason for the apparent low sensitivity of FDG-PET is the close proximity of the peripancreatic and paraaortic lymph node basins to the primary tumor, which can obscure their detection[2,16].

The important impact of FDG-PET on staging has been in its ability to identify distant metastases[17]. According to the previous reports[18], the sensitivity of FDG-PET for detecting hepatic metastases is about 70%, while that of this study was 52.6%. In particular, small lesions less than 1 cm could not be detected. It has also been reported that the sensitivity for lesions less than or greater than 1 cm is 43% and 97%, respectively[19]. Direct spread into the peritoneum is also not uncommon and often is missed on conventional imaging. However, in this study, both FDG-PET and CT failed to demonstrate a high degree of accuracy in these diagnostic analyses.

### *The prognostic significance of FDG-PET*

Some researchers have shown the SUV in FDG-PET to be an independent prognostic factor in a subpopulation of patients with pancreatic cancer. Nakata *et al*[5] showed that in patients with unresectable disease, a high SUV correlated with a shorter survival. Maemura *et al*[20] reported that pancreatic tumors with distant metastasis showed significantly higher SUV levels than tumors without metastasis. Sperti *et al*[6] also demonstrated that a high SUV (> 4.0) was associated with shorter survival. For prognostic factors for pancreatic cancer, the tumor stage and grade[21], R0 resection[21], levels of serum tumor marker[22], size of the primary lesion[3], and status of nodes metastases[3,11], have been reported. On the other hand, the increased glycolytic activity of the tumor detected by the SUV may represent tumor growth and also resemble the tumor's biological behavior[23-25]. Therefore, in this study we examined the correlation between the SUV and the patho-histological findings. The results were that the SUV did not correlate with the pTNM stage, grades, invasions to the vessels and nerve, or size of the tumor. Sperti *et al*[6] also found no difference between a high SUV (> 4.0) and a low SUV patients group in regard with TNM staging and histological grading, as shown in this study. Nevertheless, they found SUV to be an independent prognostic factor in patients with pancreatic cancer, and therefore advocated that the different biological aggressiveness of the tumor, detected by the SUV, might explain the difference in survival in patients with otherwise similar prognostic valuables. In the present study, there was a statistically significant difference in the SUV between the patients with resectable and unresectable disease. This may support the findings of previous reports which describe the SUV to be an independent prognostic factor. Therefore, although in staging FDG-PET did not perform precisely enough except for detecting bone metastases, by evaluating the SUV, FDG-PET may provide such additional information on the biological aggressiveness of the tumor, and thus play an important role in helping to select patients for subsequent surgical therapy. Some small studies reported that the tumor SUVs were useful in predicting the effectiveness of chemotherapy for unresectable pancreatic cancer[17,26,27]. However, conclusions must await further studies including larger population of patients.

In conclusion, FDG-PET was found to be useful for diagnosing pancreatic cancer. However, in staging of the disease, FDG-PET does not perform precisely enough. Although the SUV does not correlate with the patho-histological prognostic factors, it may be useful in selecting patients who should undergo the subsequent surgical treatment. Therefore, FDG-PET may play an important role in the decision making process and surgical management for patients with pancreatic cancer. However, this study suffers from the limitation of a small population of patients. Therefore, a definite conclusion may have to wait for further studies involving a larger population of patients. Furthermore, the use of image fusion with PET/CT[28,29] or with PET/MRI[30] may improve the accuracy when staging pancreatic cancer.

## COMMENTS

### *Background*

Pancreatic cancer is one of the most lethal human cancers and it continues to be a major unsolved health problem worldwide. Only patients with localized disease and

a tumor size less than 2 cm with no lymph node metastases can expect long-term survival after surgery. Therefore, increased efforts should be focusing not only on diagnosing the early stage disease, but also on staging and predicting prognosis so that unnecessary surgical exploration may be avoided. Recently, positron emission tomography (PET) has been reported to be superior to computed tomography (CT), ultrasound (US), and endoscopic US (EUS) in its sensitivity and specificity in diagnosing pancreatic cancer. Furthermore, the metabolic activity of the tumor evaluated by uptake value of fluorodeoxyglucose (FDG) (standardized uptake value: SUV) may be of prognostic significance. However, the role of PET in the management of pancreatic cancer has yet to be established.

### Research frontiers

As is described above, pancreatic cancer is one of the most lethal human cancers and it continues to be a major unsolved health problem worldwide. Despite efforts in the past 50 years, conventional treatment approaches, such as surgery, radiation, chemotherapy, or combination of these, have had little impact on the course of this aggressive neoplasm. Although recent progress in systemic chemotherapy has been reported, surgery remains the only hope for long-term survival. In surgery for pancreatic cancer, a great deal of effort has been made to expand the resection with an extended lymphadenectomy in order to improve the outcome. However, only patients with localized disease and a tumor size less than 2 cm with no lymph node metastases can expect long-term survival after surgery. Therefore, breakthrough for pancreatic cancer may be achieved in diagnosing an early stage disease.

### Innovations and breakthroughs

The present study showed that FDG-PET was useful for diagnosing pancreatic cancer. However, in staging of the disease, FDG-PET does not perform precisely enough. Although the SUV does not correlate with the patho-histological prognostic factors, it may be useful in selecting patients who should undergo the subsequent surgical treatment. Therefore, FDG-PET may play an important role in the decision making process and surgical management for patients with pancreatic cancer.

### Applications

By using FDG-PET in combination with conventional diagnostic modalities, such as computed tomography (CT), ultrasound, magnetic resonance imaging, and endoscopic pancreatography, the ability to diagnose the early stage pancreatic cancer, or to select candidates for surgery can be improved. At the same time, as the increased glycolytic activity of the tumor detected by SUV may represent tumor growth and resemble the tumor biological behavior, the SUV may provide additional information on the biological aggressiveness of the tumor.

### Terminology

FDG-PET: 18F-fluorodeoxyglucose (FDG) is a positron-emitting radio-tracer that is transported intracellularly *via* glucose transporters which are highly expressed in various cancer cells, then FDG is phosphorylated by hexokinase to FDG-6-PO4. However, further metabolism of FDG-6-PO4 is not possible in the neoplastic cells due to insufficient phosphatase levels and tracer accumulation. Therefore, FDG-PET can image cancer cells based on such specific tissue metabolism. SUV: To perform a quantitative analysis for accumulation of FDG, the standardized uptake value (SUV) is calculated in the suspected neoplastic foci. The SUV was calculated as follows:

SUV = (activity in region of interest in mCi)/(injected dose in mCi/weight in kg).

### Peer review

This is a good clinical research on the diagnosis, staging and choice of operation in patients with pancreatic carcinoma.

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*RAPID COMMUNICATION*

# Genetic changes of *p53*, K-*ras*, and microsatellite instability in gallbladder carcinoma in high-incidence areas of Japan and Hungary

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## Abstract

**AIM:** To disclose geographic differences in genetic changes involved in gallbladder carcinogenesis between two distinct high-incidence areas of Japan and Hungary.

**METHODS:** We examined 42 cases of gallbladder carcinoma: 22 Japanese and 20 Hungarian cases. *p53* mutations at exons 5 to 8 and K-*ras* mutations at codon 12 were tested by direct sequencing. Microsatellite instability was determined from fluorescent dye-labeled PCR amplifications of five-microsatellite markers (*BAT-25*, *BAT-26*, *D2S123*, *D5S346*, and *D17S250*).

**RESULTS:** Mutations of *p53* were detected in 11 of 22 Japanese cases and 6 of 18 Hungarian cases (11/22 *vs* 6/18, $P = 0.348$). Transition at CpG sites was found in none of 11 Japanese cases and 2 of 6 Hungarian cases; the difference was marginally significant (0/11 *vs* 2/6, $P = 0.110$). K-*ras* mutations were detected in only one of the Hungarian cases. Eight of 19 (42.1%) Japanese cases were MSI-high (presence of novel peaks in more than one of the five loci analyzed), whereas only 1 of 15 (6.7%) Hungarian cases was MSI-high ($P = 0.047$).

**CONCLUSION:** It appears that the *p53* mutations and MSI differ in patients with gallbladder carcinoma between two distinct high-incidence areas. Geographic variation might exist in the process of gallbladder carcinogenesis.



**Key words:** Gallbladder; Gallbladder Neoplasms; K-*ras*; Microsatellite instability; *p53*

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## INTRODUCTION

Although considerable progress has been made regarding the molecular pathogenesis of human neoplasms such as colorectal carcinoma[1,2], pancreatic carcinoma[3,4], and breast carcinoma[5,6], there is only limited information about the genetic changes involved in gallbladder carcinogenesis[7,8]. Gallbladder carcinoma shows striking geographic and ethnic variation[9,10]. The high-incidence areas are scattered throughout the world: Latin America, Eastern Europe, northern India, and Japan[9-13]. The highest mortality rate of gallbladder carcinoma in the world, 35 per 100 000 inhabitants, is found in Southern Chile[9]. Japanese standardized mortality rates for gallbladder carcinoma were world's second highest for males and fifth highest for females in 1996 with a steady increase in incidence (up to 5 per 100 000)[9,10,14]. On the other hand,

Zatonski reported that Hungarian mortality rates of this tumor were the highest in Europe (4 per 100 000 males and 7 per 100 000 females)[13]. This marked geographic variation implies that a combination of genetic and environmental etiologic factors affects the process of carcinogenesis of the gallbladder[10]. Previously, we revealed the geographic variation of gallbladder carcinogenesis between Japan and Chile (a representative of Latin American countries), both of which were known as distinct high-incidence countries, in terms of the *p53* mutational spectra[14]. Only a few other investigators also have reported such geographic and ethnic differences in genetic changes of this tumor[15-17]. Thus, there has been a paucity of evidence regarding the geographic diversity of genetic changes involved in gallbladder carcinogenesis.

The aim of this study was to disclose geographic differences in genetic changes involved in gallbladder carcinogenesis by comparing gallbladder carcinomas from two distinct high-incidence areas, Japan and Hungary (a representative country of Eastern Europe), in terms of *p53* mutations, K-ras mutations, and microsatellite instability (MSI).

## MATERIALS AND METHODS

### Tissue specimens

From 1982 to 1996, 22 patients with gallbladder carcinoma underwent a resection at Niigata University General Hospital and its affiliated institutions in Niigata Prefecture; the surgical specimens were collected and stored (Japanese cases). All the patients were Japanese. The Japanese cases in this study were identical with the Japanese cases in our previous study[14]. From 1982 to 2001, 20 patients with gallbladder carcinoma underwent a resection in hospitals in Budapest, Hungary; the surgical specimens were collected and stored (Hungarian cases) through the courtesy of a Hungarian oncologist (I.L.) and surgical pathologist (Z.S.). All the patients were residents in Budapest. Both the Japanese cases and the Hungarian cases (a total of 42 cases) formed the basis of this retrospective study. All of the patients gave informed consent for pathologically examining the specimens. No patient in this series was diagnosed with anomalous union of the pancreatic and biliary ducts (AUPBD), and had a family history suggestive of hereditary nonpolyposis colorectal cancer (HNPCC).

The surgical specimens were fixed in formalin and embedded in paraffin. One or two representative sections of each specimen were used for DNA analysis. Histopathological findings were described according to the tumor-node-metastasis (TNM) staging system[18].

### DNA preparation

Five serial slices 10-μm thick were cut from each representative histologic section and deparaffinized. Cancer tissue in each slice was dissected under microscopic guidance as described previously[14]. Non-neoplastic tissue in each slice was used as a source of constitutional DNA. DNA was extracted using a DNA Isolator PS Kit (Wako Pure Chemical Industries, Ltd., Osaka, Japan) as described previously[14].

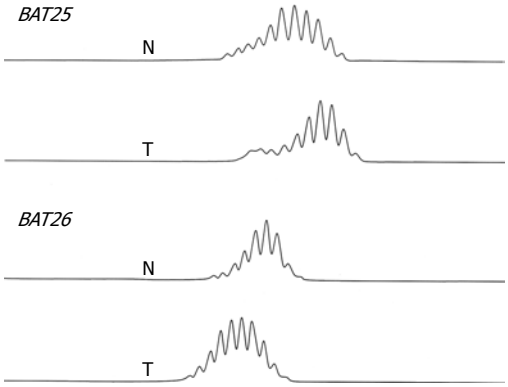


**Figure 1** Fragment pattern of case No 1 (Table 1) showing microsatellite instability at two loci (*BAT25*, *BAT26*). N: Normal DNA; T: Tumor DNA.

### Analysis of p53 mutations

*p53* mutations at exons 5 to 8 were tested by nested polymerase chain reaction (PCR) and direct sequencing as described previously[14].

### Analysis of K-ras mutations

K-*ras* mutations at codon 12 were tested by nested PCR, PCR-restriction fragment length polymorphism, and direct sequencing as described elsewhere[19].

### Analysis of MSI

Fluorescent dye-labeled PCR amplification was performed using the five microsatellite markers (*BAT-25*, *BAT-26*, *D2S123*, *D5S346*, and *D17S250*) recommended by the National Cancer Institute workshop[20,21]. Fluorescent dye-labeled and unlabeled primers were obtained (Applied Biosystems Japan Ltd., Tokyo, Japan); the 5' oligonucleotide was end-labeled with 6FAM (*BAT-25*, *D2S123*), VIC (*BAT-26*, *D17S250*), or NED (*D5S346*) fluorescent dyes. Amplification by PCR was performed using the Temp Control System PC-700 (ASTEC Co., Ltd., Fukuoka, Japan) in 30-μL reaction volumes containing 100 ng of DNA, 0.75 U of AmpliTaq Gold (Applied Biosystems Japan Ltd.), and 10 pmol of each primer. The cycling profile was: denaturation at 95℃ for 2 min, annealing at 55℃ for 40 s and extension at 72℃ for 40 s followed by a 7-min final extension step. A 1.0-μL aliquot of each fluorescent dye-labeled PCR product was combined with 12 μL of formamide and 0.5 μL of the GENESCAN 350 [ROX] size standard (Applied Biosystems Japan Ltd.) and analyzed on an ABI PRISM 310 Genetic Analyzer using GeneScan Analysis Software (Applied Biosystems Japan Ltd.). MSI experiments were repeated twice to exclude the possibility of a false positive result due to PCR amplification artifacts in an independent PCR reaction. The sequencing results were verified by a second independently generated PCR product.

The presence of MSI was determined from the PCR amplifications of the five-microsatellite markers. Microsatellite instability was defined as the presence of novel peaks that were not found in non-neoplastic tissue (Figure 1). A tumor was defined as having high microsatellite instability (MSI-high) if more than one of

the five loci analyzed showed unequivocal instabilities; a tumor was defined as having low microsatellite instability (MSI-low) if only one locus showed instability; and a tumor was defined as microsatellite stable if no microsatellite instability was found. In this study, MSI-low tumors and microsatellite-stable tumors were categorized together into one group (MSI-low/none), as proposed by recent authors[16,22].

### Statistical analysis

Fisher's exact test was used to compare the frequencies of genetic alterations between the two groups. All statistical evaluations were performed using the SPSS 11.5J software package (SPSS Japan Inc., Tokyo, Japan). All tests were two-sided and a $P$ value $< 0.05$ was considered statistically significant.

## RESULTS

### p53 mutation

Mutations of *p53* at exons 5 to 8 were detected in 11 of 22 (50.0%) Japanese cases and 6 of 18 (33.3%) Hungarian cases ($P = 0.348$; Table 1). Among the 17 cases with *p53* mutations, transversion was found in 4 of 11 Japanese cases and 1 of 6 Hungarian cases ($P = 0.600$). Transition at CpG sites was found in none of 11 Japanese cases and 2 of 6 Hungarian cases; the difference was marginally significant ($P = 0.110$).

### K-ras mutation

K-*ras* mutations at codon 12 were detected in none of 22 Japanese cases and 1 of 20 Hungarian cases ($P = 0.476$) (Table 1).

### MSI

Eight of 19 (42.1%) Japanese cases were MSI-high, whereas only one of 15 (6.7%) Hungarian cases was MSI-high; the difference was statistically significant ($P = 0.047$; Table 1).

### Association between p53 mutation and MSI in gallbladder carcinoma

Both *p53* mutation and MSI were analyzed successfully in 34 cases: 19 Japanese cases and 15 Hungarian cases (Table 1). When the cases were stratified into Japanese cases and Hungarian cases, *p53* mutations were not associated significantly with MSI in both groups. In all 34 cases, *p53* mutations were associated significantly with MSI (Table 2; $P = 0.033$).

## DISCUSSION

Although earlier epidemiologic studies have suggested that the incidence of gallbladder carcinoma varies geographically or ethnically[9,10], there is a paucity of evidence regarding the geographic diversity of molecular changes associated with this tumor[7,8]. This prompted us to conduct the current study. In 1998, we reported that the *p53* mutational spectra of gallbladder carcinoma differed considerably between Japan and Chile, both of which were known as high-incidence countries[14]. The current study first demonstrates that the process of gallbladder carcinogenesis differs in terms of *p53* mutations and microsatellite instability between two distinct high-incidence areas: Japan and Hungary (a representative country of Eastern Europe).

Reported prevalences of *p53* mutation for gallbladder carcinoma ranged from 31% to 70%[14,23-26], suggesting that *p53* mutation plays an important role in the development of this tumor[8]. The prevalences of *p53* mutation in this series (50% in Japanese cases and 33% in Hungarian cases) are comparable with the reported figures. In various malignancies, endogenous carcinogenesis is characterized by transitions at CpG sites, whereas transversions imply the presence of an exogenous mutational process[27-29]. In our previous study, the prevalence of transitions at CpG sites was significantly higher in the Chilean cases than in the Japanese cases[14]. In the current study, there was a trend toward a higher prevalence of transitions at CpG sites in the Hungarian cases. Taken together, these observations suggest that an endogenous mutational process contributed considerably to carcinogenesis of the gallbladder in the Chilean and Hungarian cases. In contrast, considering that there were no transitions at CpG sites and four transversions in the Japanese cases, it appears that exogenous mutations often happen during the process of carcinogenesis in the Japanese cases. Therefore, geographic variation might exist in carcinogenesis of the gallbladder among three distinct areas.

Reported prevalences of K-*ras* mutation for gallbladder carcinoma without AUPBD ranged from 0% to 17%[16,30-33], suggesting that frequency of K-*ras* mutation is relatively low in gallbladder carcinoma without AUPBD. This is consistent with our results: K-*ras* mutation was found in only one of the Hungarian cases. Taken together, most gallbladder carcinoma appears to develop from a K-*ras*-independent pathway.

Microsatellite instability, which represents replication errors, results from DNA mismatches due to environmental or hereditary factors and leads to genomic instability[34,35]. Reported prevalences of MSI-high for sporadic gallbladder carcinoma range from 0% to 10%[15,16,36-38], whereas the prevalence was high (42.1%) in our cases. This suggests that environmental or hereditary factors contribute to carcinogenesis of the gallbladder in some of our cases. In the current study, the prevalence of MSI-high was significantly higher in the Japanese cases than in the Hungarian cases. Considering that there were no patients with HNPCC in this series, the above finding suggests that the high prevalence of MSI-high in the Japanese cases may be due to environmental factors, which remain unknown.

There are inverse relationships between *p53* mutations and MSI in colorectal cancer[39,40]. Regarding gallbladder carcinoma, earlier authors have failed to identify such relationships between *p53* mutations and MSI[16,38]. In this study, *p53* mutations were associated significantly with MSI in all cases. When the cases were stratified into Japanese cases and Hungarian cases, no such associations between *p53* mutation and MSI were found, probably due to the small sample size. Taken together, the above observations suggest that the pathway of carcinogenesis differs between the gallbladder and the colon and rectum.

The current study has limitations. Firstly, it was a

**Table 1 Genetic alterations observed in gallbladder carcinomas from Japan and Hungary**

| Case No | Age (yr) | Sex | Histology[1] | | *p53* mutation | | K-*ras* mutation | Microsatellite instability |
|---|---|---|---|---|---|---|---|---|
| | | | Type | Grade | Exon/codon | Base change | Base change | |
| Japanese cases | | | | | | | | |
| 1 | 71 | F | PAP | G1 | None | - | None | BAT25, BAT26 |
| 2 | 74 | F | AD | G1 | Exon 5/codon 132 | AAG to GAG | None | BAT25, D2S123 |
| 3 | 62 | F | PAP | G1 | None | - | None | BAT25 |
| 4 | 76 | F | AD | G1 | Exon 6/codon 193 | CAT to AAT | None | BAT25, BAT26 |
| 5 | 78 | F | AD | G2 | Exon 5/codon 140 | ACC to ATC | None | ND |
| | | | | | Exon 5/codon 166 | TCA to ACA | | |
| 6 | 70 | F | AD | G1 | Exon 8/codon 276 | GCC to CCC | None | None |
| 7 | 71 | F | AD | G1 | None | - | None | BAT26, D2S123 |
| 8 | 79 | F | AD | G1 | None | - | None | None |
| 9 | 66 | F | PAP | G1 | None | - | None | None |
| 10 | 60 | F | PAP | G1 | None | - | None | None |
| 11 | 80 | M | PAP | G1 | None | - | None | BAT26 |
| 12 | 63 | M | PAP | G1 | Exon 8/codon 294 | GAG to GAA | None | BAT25, BAT26 |
| 13 | 49 | F | PAP | G1 | None | - | None | D2S123 |
| 14 | 71 | M | AD | G2 | Exon 8/codon 280 | AGA to AAA | None | BAT25, D2S123 |
| 15 | 70 | M | AD | G1 | Exon 7/codon 238 | TGT to CGT | None | ND |
| 16 | 53 | M | AD | G1 | None | - | None | None |
| 17 | 79 | F | AD | G3 | Exon 8/codon 271 | GAG to AAG | None | None |
| 18 | 62 | M | AD | G3 | Exon 6/codon 205 | TAT to TGT | None | None |
| 19 | 61 | M | AD | G1 | None | - | None | D17S250, D2S123 |
| 20 | 69 | M | AD | G2 | Exon 7/codon 231 | ACC to ATC | None | BAT25, D17S250 |
| 21 | 58 | F | AD | G2 | Exon 5/codon 160 | ATG to GTG | None | ND |
| | | | | | Exon 6/codon 220 | TAT to AAT | | |
| 22 | 76 | F | AD | G3 | None | - | None | None |
| Hungarian cases | | | | | | | | |
| 23 | 71 | M | AD | G2 | Exon 7/codon 249 | AGG to ACG | None | BAT26 |
| 24 | 69 | F | AD | G3 | Exon 6/codon 213 | CAG to CGG | None | None |
| 25 | 69 | F | AD | G2 | None | - | None | None |
| 26 | 76 | F | AD | G2 | None | - | None | None |
| 27 | 61 | F | AD | G3 | None | - | None | None |
| 28 | 58 | F | AD | G3 | None | - | None | BAT25 |
| 29 | 69 | F | AD | G3 | None | - | None | None |
| 30 | 75 | F | PAP | G1 | None | - | None | None |
| 31 | 66 | F | AD | G3 | None | - | None | None |
| 32 | 65 | F | AD | G1 | Exon 6/codon 219 | CCC to TCC | None | ND |
| 33 | 61 | F | AD | G3 | ND | - | None | ND |
| 34 | Unknown | F | PAP | G1 | Exon 5/codon 138 | GCC to GCT | None | BAT25, D17S250 |
| | | | | | Exon 5/codon 167 | CAG to CAA | | |
| | | | | | Exon 5/codon 170 | ACG to ACA | | |
| 35 | Unknown | F | PAP | G1 | Exon 5/codon 158 | CGC to TGC | GGT to AGT | ND |
| | | | | | Exon 6/codon 223 | CCT to TCT | | |
| 36 | Unknown | F | AD | G3 | Exon 6/codon 210 | AAC to AAT | None | ND |
| | | | | | Exon 7/codon 228 | GAC to AAC | | |
| 37 | Unknown | F | AD | G2 | ND | - | None | ND |
| 38 | 53 | F | AD | G3 | None | - | None | None |
| 39 | 72 | Unknown | AD | G1 | None | - | None | None |
| 40 | 69 | M | AD | G1 | None | - | None | BAT26 |
| 41 | 57 | F | AD | G1 | None | - | None | None |
| 42 | 74 | F | AD | G1 | None | - | None | BAT25 |

[1]According to the tumor-node-metastasis (TNM) staging system[18]. PAP: Papillary carcinoma; AD: Adenocarcinoma; ND: Not detected; G1: Well differentiated; G2: Moderately differentiated; G3: Poorly differentiated.

**Table 2 Association between *p53* mutation and MSI in gallbladder carcinoma**

| | | MSI-high | MSI-low/none | *P* |
|---|---|---|---|---|
| *p53* mutation | Present | 6 | 5 | 0.033 |
| | Absent | 3 | 20 | |

MSI: Microsatellite instability.

retrospective analysis of a small number of patients. Secondly, DNA preparation from paraffin-embedded tissue sections was unsuccessful in some patients. Thirdly, only limited clinical information was available in the Hungarian cases to protect the patients' privacy. To our knowledge, however, this study demonstrates more clearly the geographic diversity of gallbladder carcinogenesis than earlier reports[15-17].

In conclusion, it appears that the *p53* mutations and MSI differ in patients with gallbladder carcinoma between two distinct high-incidence areas. Exogenous mutations

and unknown environmental factors may play roles in gallbladder carcinogenesis in the Japanese cases, whereas the Hungarian cases are characterized by an endogenous mutational process. Thus, geographic variation might exist in the process of gallbladder carcinogenesis.

## ACKNOWLEDGMENTS

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## COMMENTS

### Background

Although considerable progress has been made regarding the molecular pathogenesis of human neoplasms, there is only limited information about the genetic changes involved in gallbladder carcinogenesis. Gallbladder carcinoma shows striking geographic and ethnic variation, however there is a paucity of evidence regarding the geographic diversity of molecular changes associated with this tumor.

### Research frontiers

In 1998, we reported that the *p53* mutational spectra of gallbladder carcinoma differed considerably between Japan and Chile, both of which were known as high-incidence countries. Only a few other investigators also have reported such geographic and ethnic differences in genetic changes of this tumor. The aim of this study was to disclose geographic differences in genetic changes involved in gallbladder carcinogenesis by comparing gallbladder carcinomas from two distinct high-incidence areas, Japan and Hungary.

### Innovations and breakthroughs

The current study first demonstrates that the *p53* mutations and MSI differ in patients with gallbladder carcinoma between two distinct high-incidence areas: Japan and Hungary. Exogenous mutations and unknown environmental factors may play roles in gallbladder carcinogenesis in the Japanese cases, whereas the Hungarian cases are characterized by an endogenous mutational process. Thus, geographic variation might exist in the process of gallbladder carcinogenesis.

### Applications

Gallbladder carcinoma is a highly lethal disease with a poor prognosis. Therefore, it would be very beneficial to identify the molecular mechanism responsible for this condition. Advances in the understanding of the genetic changes of this tumor will help in understanding the pathogenesis of this miserable disease.

### Terminology

Microsatellite instability (MSI) is caused by a failure of the DNA mismatch repair system to repair errors that occur during the replication of DNA and is characterized by the accelerated accumulation of single nucleotide mutations and alterations in the length of simple, repetitive microsatellite sequences that occur ubiquitously throughout the genome. MSI is seen in most HNPCC tumors and proportion of nonhereditary colorectal tumors. The presence of MSI in tumor tissue is associated with certain unique clinical and pathological characteristics.

### Peer review

This study aimed to determine the difference of some genetic changes involved in gallbladder carcinogenesis in high-prevalence areas of Japan and Hungary. This is a well written paper and has a nice finding. While it is simply an observational study, I think it is an important observation.

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*RAPID COMMUNICATION*

# Midkine secretion protects Hep3B cells from cadmium induced cellular damage

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## Abstract

**AIM:** To evaluate role of midkine secretion during Cadmium (Cd) exposure in the human hepatocyte cell line Hep3B cells.

**METHODS:** Different dosages of Cd (0.5-1-5-10 μg/mL) were applied to Hep3B cells and their effects to apoptosis, lactate dehydrogenase (LDH) leakage and midkine secretion were evaluated as time dependent manner. Same experiments were repeated with exogenously applied midkine (250-5000 pg/mL) and/or 5 μg/mL Cd.

**RESULTS:** Cd exposure induced prominent apoptosis and LDH leakage beginning from lower dosages at the 48$^{th}$ h. Cd induced midkine secretion with higher dosages ($P$ < 0.001), (control, Cd 0.5-1-5-10 μg/mL respectively: 1123 ± 73, 1157 ± 63, 1242 ± 90, 1886 ± 175, 1712 ± 166 pg/mL). Exogenous 500-5000 pg/mL midkine application during 5 μg/mL Cd toxicity prevented caspase-3 activation (control, Cd toxicity, 250, 500, 1000, 2500, 5000 pg/mL midkine+ Cd toxicity, respectively: 374 ± 64, 1786 ± 156, 1545 ± 179, 1203 ± 113, 974 ± 116, 646 ± 56, 556 ± 63 cfu) LDH leakage and cell death in Hep3B cells ($P$ < 0.001).

**CONCLUSION:** Our results showed that midkine secretion from Hep3B cells during Cd exposure protects liver cells from Cd induced cellular damage. Midkine has anti-apoptotic and cytoprotective role during Cd toxicity. Further studies are needed to explain the mechanism of midkine secretion and cytoprotective role of midkine during Cd exposure. Midkine may be a promising therapeutic agent in different toxic hepatic diseases.



**Key words:** Cadmium; Midkine; Hepatocyte; Apoptosis; Caspase-3; Lactate dehydrogenase

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## INTRODUCTION

Cadmium (Cd) exposure occurs widely in the general population, especially low-level chronic exposure through smoking and dietary sources, but it is known as one of the most toxic environmental and industrial pollutants. Cd accumulates in the body because of slow excretion[1]. Cd causes toxicity in different organs. Acute and chronic Cd exposure mostly results in hepatotoxicity[2]. It seems that the level of damage depends on the dosage and duration of Cd application. Exposure of cells to toxic chemicals is known to up-regulate the expression of a number of stress proteins and results in activation of apoptotic pathways and consequently cellular damage. *In vivo* and *in vitro* studies showed that inflammation and oxidative damage are main mechanisms of Cd induced toxicity[3-6]. Cd induces mitochondria-dependent apoptotic pathways where caspase-3 and caspase-9 are activated[7].

Midkine is a heparin binding growth factor. It takes part in cancer and inflammation[8]. Although midkine is a mitogenic factor during carcinogenesis, it plays a critical role in ischemia induced inflammatory damage[9]. It was demonstrated that midkine acts as an antiapoptotic factor in HepG2 cells; furthermore, midkine suppressed the activity of caspase-3, which plays a significant role in the apoptotic pathway. Pretreatment with midkine prevents tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) mediated apoptosis in the HepG2 cells[10]. TRAIL alone triggered massive apoptosis accompanied by caspase activation in tissue explants from patients with liver steatosis or hepatitis C viral

infection[11]. TNF-alpha, released from nonparenchymal cells as well as associated cytokines, are responsible for clinical expression and tissue damage observed with cadmium-induced hepatotoxicity[12]. It was shown that midkine expression was upregulated in a marine gastropod limpet patella caerulea after they were exposed to sublethal doses of Cd[13]. But, whether midkine takes part in Cd induced mechanisms in human cells is still unknown. In this study we aimed to evaluate effects of Cd induced midkine secretion in the human hepatocyte cell line Hep3B cells, and its effects to cellular proliferation, apoptosis and biochemical parameter of cellular integrity during Cd exposure.

## MATERIALS AND METHODS

### Cell lines, chemicals and materials

Human hepatoma cell line Hep3B cells were obtained from the ATCC. Cells were cultured in Roswell Park Memorial Institute (RPMI)-1640 medium (PAA, Austuria), supplemented with fetal calf serum (FCS), (PAA, Austuria), L-glutamine (Sigma, USA), streptomycin (Sigma, USA) and penicillin (Sigma, USA). Cadmium ($CdCl_2$, Sigma Chemical Co., St. Louis, MO, USA) was dissolved in water, sterilized by 0.22 μm pore size cellulose acetate membrane filters, and added to cultures at the indicated time and concentrations. $CdCl_2$ toxicity was studied in Hep3B cell line. Human recombinant midkine was obtained from Peprotech (UK). Cell counts were tested by 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide (MTT, Sigma, USA). For evaluation of apoptosis caspase-3 levels were measured by a flourometric kit (Biotium, USA). Lactate Dehydrogenase (LDH) level was measured with a kit using an automatic multianalyzer (Roche; P800). Midkine levels were measured by an ELISA development kit (Peprotech,UK).

### Cell culture and experimental protocol

The human hepatoma cell line Hep3B was cultured in RPMI-1640 medium, supplemented with 10% v/v fetal calf serum, 2 mmol/L L-glutamine, streptomycin (100 μg/mL) and penicillin (100 IU/mL) in a humidified atmosphere containing 5% $CO_2$ at 37℃. One day before the experiments, cells were seeded on 96-well microtitre plates (Nunc, Denmark) at $2 \times 10^5$ cells/mL.

Depending on the groups, different concentrations of cadmium (0.5-1-5-10 μg/mL) and midkine (250, 500, 1000, 2500, 5000 pg/mL) were added to medium for 48 h. For evaluation of the effects of midkine during cadmium toxicity; cadmium was used at a dosage of 5 μg/mL. $n = 6$, for every experimental group.

LDH and caspase-3 levels were evaluated from cadmium and/or midkine treated cells at the 48$^{th}$ h. MTT was measured at the 2$^{nd}$, 24$^{th}$ and 48$^{th}$ h. Midkine was measured from supernatants. After supernatants were removed cell surface was washed with sterile phosphate buffered saline (PBS) and cells were harvested with lysis solution and caspase-3 levels of groups were measured from cell lysates. LDH measurement was done from both of the supernatant and cell lysates.

### Evaluation of cellular proliferation or death

MTT, a colorimetric assay based upon the ability of living cells to reduce MTT into formazan, was used for evaluation of the effects of dose and time dependent effects of cadmium and midkine on cellular death or proliferation (2$^{nd}$, 24$^{th}$, 48$^{th}$ h). Cell number % was calculated as ratio of cell number of effected group *vs* control group × 100 at the determined hour.

### Biochemical determination of cell death

Hep3B cells were plated in 96 multiwell cell culture plates as $3 \times 10^5$ cells/mL. LDH is normally present in the cytosol of hepatocytes. In response to cell damage LDH is released from the cells. Therefore, to determine cell death, we measured secreted and intracellular LDH levels and calculated % released LDH at the 48$^{th}$ h for each group. To do this, the medium was collected to measure enzyme activities. The adherent cells were lysed. Both medium and cell lysates were used for quantitative determination of LDH activity (IU/L) which was performed with an automatic multianalyzer (Roche) using a kit (Roche). Released enzyme fractions for each sample were calculated as the ratio of enzyme present in the medium *vs* the sum of the levels of same enzyme in the supernatant and in the cells.

### Measurement of apoptosis

**Caspase-3 levels:** The presence of apoptosis was determined by caspase-3 levels. Equal numbers of cells were used for caspase-3 level measurements. Cells were lysed with assay buffer (50 mmol/L HEPES, pH 7.4, 100 mmol/L NaCl, 0.1% CHAPS, 10 mmol/LM DTT, 2 mmol/L EDTA, 2 mmol/L EGTA, Triton X-100, 0.1%). Caspase-3 levels were measured by DEVD-R110 Fluorometric HTS Assay Kit from cell lysates. The fluorogenic substrate $(Ac\text{-}DEVD)_2$-R110 was used for this assay. It is completely hydrolyzed by the enzyme in two successive steps. Cleavage of the first DEVD peptide results in the monopeptide Ac-DEVD-R110 intermediate, which has absorption and emission wavelengths similar to those of R110 ($\lambda_{abs}/\lambda_{abs}$ = 496/520 nm), but has only about 10% the fluorescence of the latter. Hydrolysis of the second DEVD peptide releases the dye R110, leading to a substantial fluorescence increase.

Equal volumes of sample and caspase-3 detection buffer were added to assay plate, and then incubated at 37℃ for 1 h in an incubator. Results were read with a fluorometer at 470 nm excitation filter and 520 nm emission filters. R110 was used for generating a standard curve to calculate amount of substrate conversion.

### Statistical analysis

Results of the experiments were analyzed by One Way ANOVA, followed by a multiple comparison test using SPSS 10.0. $P < 0.05$ was accepted as statistically significant. Results were given as mean ± SEM.

## RESULTS

### Cell proliferation and toxicity

We characterized the concentration-dependent cytotoxic effect of Cd on human hepatocyte cell line as a function of time. Cadmium exposure decreased living cell number depending on the dosage. Minimal cytotoxic (< 5%) effect was seen at the 0.5 μg/mL dosage. As shown in Figure 1,

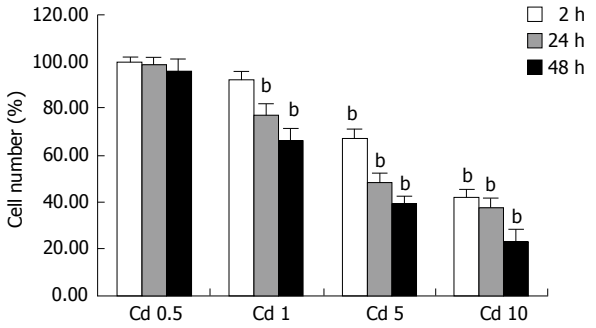


**Figure 1** Cell death (%) was determined at 2nd, 24th and 48th h by the MTT assay. Cd exposure induced prominent cell death in Hep3B hepatocytes with dose and time dependent manner. Data are from 6 independent experiments for each condition. Data are presented as mean ± SEM. $^bP < 0.001$ *vs* control group.

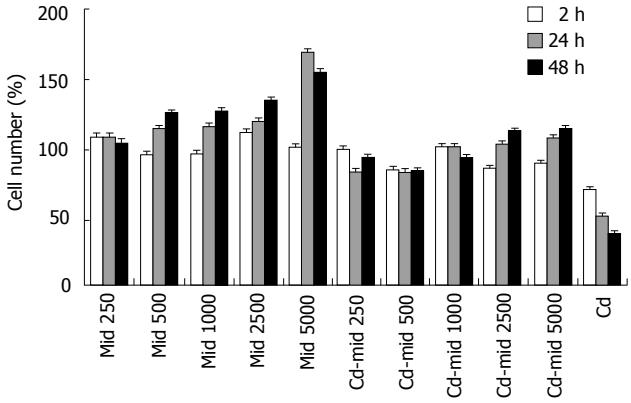


**Figure 2** Cell number (%) was determined by MTT assay following 2nd, 24th and 48th h exposure to 250-5000 pg/mL and/or 5 μg/mL Cd. Midkine treatment increased cell proliferation in Hep3B hepatocytes with dose and time dependent manner. Data are presented as mean ± SEM.

cell exposure to 1 μg/mL Cd for up to 2 h only slightly affected cell viability as revealed by MTT measurements compared to control values estimated in untreated cells, but it becomes apparent at the 24th and 48th h. Cd exposure caused cellular damage in a dose and time dependent manner in the Hep3B cell line. Cytotoxicity was more prominent with higher dosages at the 24th and 48th h ($P < 0.001$, Figure 1). Regarding to these data, 5 μg/mL $CdCl_2$ concentration which with moderate-high toxic impact was chosen for subsequent experiments with different dosages of midkine.

Midkine treatment caused proliferation of Hep3B cells in a dose and time dependent manner compared to control group. The highest increase in cell number was at the 48th h and 5000 pg/mL midkine concentration ($P < 0.001$, Figure 2). Midkine treatment during Cd toxicity prevents cell death, even with the lowest dosages ($P < 0.001$, Figure 2).

### *Determination of apoptosis*

Increased apoptosis was seen in Cd treated cells, which was confirmed with increased caspase-3 levels. Lowest dosage of Cd application did not increase caspase-3 levels compared to untreated cells. Activation of caspase-3 started at the 1 μg/mL Cd dosage ($P < 0.001$, Figure 3).

Midkine treatment decreased caspase-3 levels in the

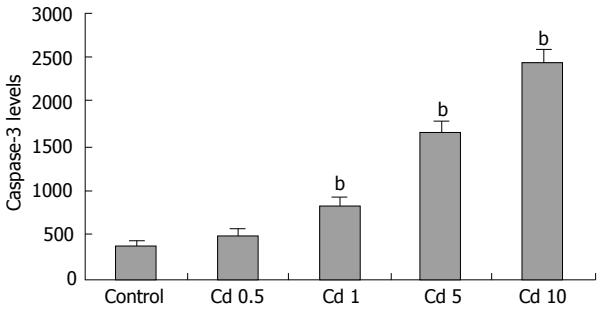


**Figure 3** Caspase-3 levels were measured following a 48 h exposure to 0.5-10 μg/mL Cd. Data were given as mean ± SEM. $^bP < 0.001$ *vs* control group.

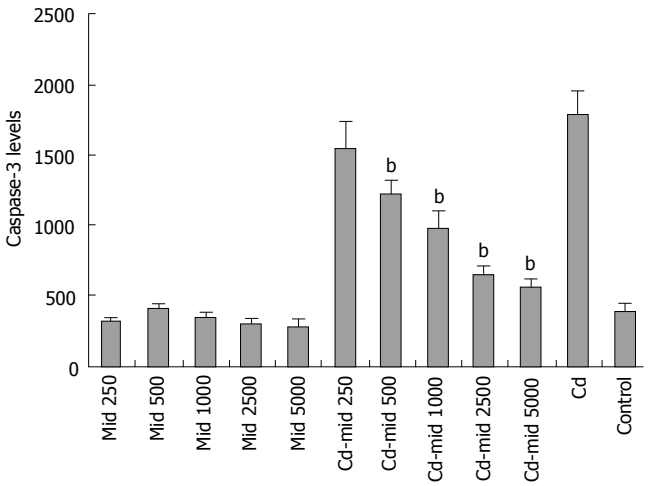


**Figure 4** Caspase-3 levels were measured following a 48 h of exposure to 250-5000 pg/mL Midkine and/or 5 μg/mL Cd. Data are presented as mean ± SEM. $^bP < 0.001$ *vs* control group.

Hep3B cells. It prevents Cd induced apoptosis prominently starting from 500 pg/mL concentration of midkine application ($P < 0.001$, Figure 4).

### *Cytotoxic effects of Cd in the hepatocytes*

Incubation of Hep3B cells with Cd resulted in cytotoxicity as assessed by LDH released into the incubation media. LDH release in the Hep3B cells to media started at the 1 μg/mL Cd dosage (Figure 5).

Midkine treatment at the same time with 5 μg/mL Cd exposure decreased LDH release in the Hep3B cells (Figure 6).

### *Midkine secretion*

Measurable basal midkine secretion was found in the Hep3B cells under normal conditions. Cd treatment induced midkine secretion in the Hep3B cells in a dose dependent manner (Figure 7).

## DISCUSSION

Acute/chronic Cd exposure mostly results in hepatotoxicity, where it is a good model to study toxic substance-induced liver damage[2]. Midkine family has strong anti-apoptotic function so they are obviously considered non specific (i.e., for Cd) mechanisms of defence[10]. Intense midkine expression has also been found in increased various human tumors and level of midkine expression correlates

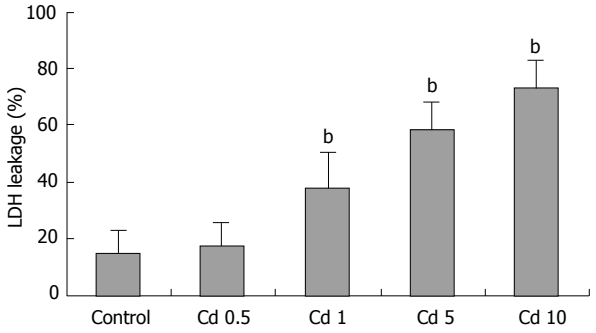


**Figure 5** Cd induced cytotoxicity at the 48th h of experiment determined by % LDH released to medium. Starting from the 1 μg/mL dosage Cd treatment caused prominent LDH release from hepatocytes at the end of 48th h ($P < 0.001$). Data are presented as mean ± SEM. $^bP < 0.001$ *vs* control group.

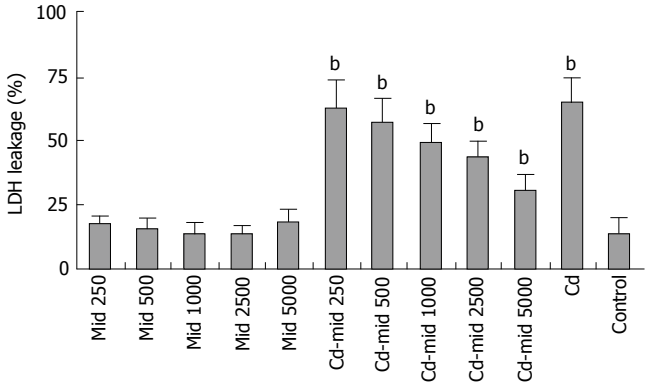


**Figure 6** Effects of 48 h midkine (250-5000 pg/mL) and/or 5 μg/mL Cd treatment on the LDH leakage in the Hep3B cells. Data are presented as mean ± SEM. $^bP < 0.001$ *vs* control group.

negatively with the patients' prognosis[14,15]. Furthermore, midkine accumulation is noted in senile plaques in the brains of Alzheimer's disease patients[16]. Midkine is expressed around the damaged neuronal site after cerebral infarction[17], suggesting a role for midkine in tissue repair.

The results of the present study supports the idea that Cd exposure causes cytotoxicity and apoptosis in the Hep3B cells. In both time and dose-response studies, LDH leakage, which is very important parameter to detect hepatocellular integrity, was greater in Cd treated cells. These effects were more prominent at the 48th h. During Cd exposure, activation of caspase-3 was detected in Hep3B cells, suggesting a caspase-dependent pathway is involved in Cd toxicity. Cd can upregulate the expression of a number of genes that produce products that can detoxify Cd and/or repair Cd induced lesions. Our studies showed that midkine is one of them. The induction pathways or receptors of midkine expressed by Cd exposure is still unclear. Midkine is multifunctional heparin-binding growth factor and cytokine and has anti-apoptotic and cell-protecting activities[8]. Untreated Hep3B cells have also a basal midkine secretion. In our study, midkine treatment decreased apoptosis and increased cellular proliferation in a dose and time dependent manner. It has cytoprotective, anti-apoptotic effects against Cd toxicity in Hep3B cells. It seems that midkine is produced endogenously and released in to medium as a defense mechanism

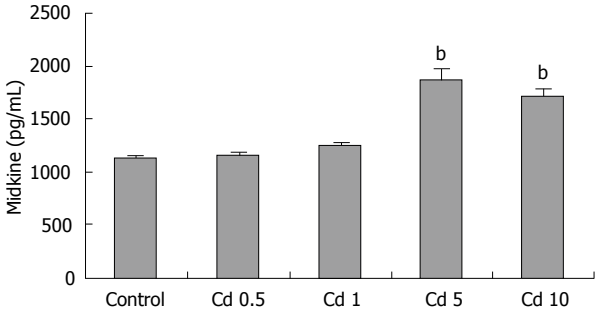


**Figure 7** Effects of 0.5-10 μg/mL Cd treatment on midkine secretion in the Hep3B cells. With 0.5 and 1 μg/mL Cd exposure we obtained similar midkine secretion as untreated cells. Midkine secretion was highest as a response to 5 μg/mL Cd treatment dosage. Data are presented as mean ± SEM. $^bP < 0.001$ *vs* control group.

of Hep3B cells against Cd toxicity. Among midkine receptors, receptor-type protein tyrosine phophatase z (PTP z) has been studied extensively. Midkine stimulates phosphorylation of specific members of the JAK/STAT pathway, namely JAK1, JAK2, and STAT1α [18,19]. In addition, low density lipoprotein receptor-related protein (LRP) has also been identified as a receptor[20]. The midkine receptor is considered to be a molecular complex containing these proteins. The downstream signaling systems of these receptors include ERK, which participates in the reduction of necrotic and apoptotic cell death[21]. Internalization of midkine in to cell and nuclear targeting is important for its antiapoptotic function[22]. Activation of these receptors and intracellular pathways might take part in cytoprotective effects of midkine during Cd toxicity. Human and experimental studies have shown that apoptosis plays a role in hepatocyte death in alcoholic liver disease and nonalcoholic steatohepatitis and apoptosis levels correlate with the severity of the liver disease[23-26]. LDL receptor-related protein (LRP) is another midkine receptor. LRP is important for lipid and lipoprotein uptake to cells[27]. Lipid profiles of steatohepatitis patients were found disturbed[28]. It was shown that midkine takes part in the inflammatory and repair processes after partial hepatectomy. They suggested that midkine is beneficial for liver regeneration[29]. ERK, JNK signal pathways disturbed during Cd toxicity are activated by midkine[18,19,22,30]. Beneficial effects of exogenous midkine to minimize Cd induced damage would provide a new perspective for innovation in the treatment of Cd intoxications and in NonAlcoholic Fatty Liver Disease (disease near exclusively characterized by apoptotic process), in Drug Induced Liver Injury and in the combined form, illnesses far long more evident than Cd intoxication in the every day practice of gastroenterologists and hospitals. But further acute and chronic *in vitro* and *in vivo* studies are needed to evaluate which intracellular pathway(s) is activated during these processes.

## COMMENTS

### *Background*

Acute/chronic Cadmium (Cd) exposure mostly results in hepatotoxicity, where it is a good model to study toxic substance-induced liver damage. Midkine is expressed around the damaged tissues after ischemic damage and suggesting a role for midkine in tissue repair. Its expression has been found in tumors and level

of midkine expression correlates negatively with the patients' prognosis. Midkine is cytoprotective and has anti-apoptotic effect.

### Research frontiers

Beneficial effects of exogenous midkine to minimize Cd induced damage would provide a new perspective for innovation in the treatment of Cd intoxications and other liver diseases.

### Innovations and breakthroughs

Cd is well known environmental toxic substance which mainly damages liver. In this study we showed that Cd treatment induces midkine secretion from hepatocytes. Midkine might have protective role during Cd toxicity.

### Applications

These finding may be used in the different liver diseases such as alcoholic, toxic and non- alcoholic liver disease models.

### Peer review

In this experimental *in vitro* study, the authors showed that midkine secretion from Hep3B cells during Cd exposure protects liver cells from Cd induced cellular damage. Midkine has anti-apoptotic and cytoprotective role during Cd toxicity. Midkine may be a promising therapeutic agent in different toxic hepatic diseases.

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*RAPID COMMUNICATION*

# Effects of primary suture and fib sealant on hemostasis and liver regeneration in an experimental liver injury

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## Abstract

**AIM:** To investigate the effects of fibrin sealant on hemostasis and liver regeneration and intra-abdominal adhesions in an experimental liver injury.

**METHODS:** Thirty-six Wistar rats were randomly divided into primary suture group ($n$ = 15), fibrin sealant group ($n$ = 15) and control group ($n$ = 6). A wedge resection was performed on the left lobe of the liver. In primary suture group, liver was sutured using polypropylene material, while fibrin glue was administrated on the liver surface in fibrin sealant group.

**RESULTS:** More intra-abdominal adhesions were observed in the primary suture group compared to the fibrin sealant group on 3rd (2.50 ± 0.5 *vs* 0.25 ± 0.5, $P$ = 0.015), 10th (2.75 ± 0.5 *vs* 0.50 ± 0.6, $P$ = 0.06) and 20th (1.75 ± 0.5 *vs* 0.70 ± 0.5, $P$ = 0.015) postoperative days. Histopathological scores were better in the fibrin sealant group in comparison with the primary suture group on 3rd (8.75 ± 0.5 *vs* 6.75 ± 0.5, $P$ = 0.006), 10th (7.50 ± 1.0 *vs* 5.5 ± 0.6, $P$ = 0.021) and 20th (6.40 ± 1.7 *vs* 3.20 ± 1.6, $P$ = 0.025) postoperative days.

**CONCLUSION:** Out data suggest that fibrin sealant is preferred over primary suture in appropriate cases including liver trauma since it causes less intra-abdominal adhesions while allowing shorter hemostasis time as assessed in experimental liver trauma.



**Key words:** Liver; Trauma; Fibrin sealant; Hemostasis; Regeneration

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## INTRODUCTION

Liver injuries occur as a result of blunt and penetrating traumas and rarely due to some iatrogenic reasons. In blunt abdominal traumas, liver turns out to be the organ that mostly gets injured, with mortality rates varying from 10% to 15%. Eighty percent of the liver injuries are formed subsequent to penetrating wounds caused by fire guns or piercing, incisive tools. Acute bleedings and operative complications cause an increase in mortality and morbidity rates[1]. Fibrin glue has been used in a wide selection of surgical fields, such as thorax surgery[2-4], and otolaryngology[5], and neurosurgery[6], and cardiovascular surgery[7]. Besides this, in general surgery application of fibrin glue has been made for treatment[8,9] of anal fistulas and repair of inguinal hernia. Fibrin sealant can be used as a safe and appropriate treatment technique in liver injuries. However, its effects on liver regeneration and development of intra-abdominal adhesions yet remain unclear. In this experimental study, the early and late effects of the reparation techniques employing primary sutures and fibrin glues on an experimental trauma model are evaluated.

## MATERIALS AND METHODS

The study was performed at the animal laboratory of Ministry of Health Ankara Training and Research Hospital, after obtaining an approval from the Ethics Committee. For the purposes of the study, 36 male albino Wistar rats were selected from ages varying between 18 to 20 wk and an average body weight of 160 g. Animals were fed standard rodent food and water. They were left hungry for 24 h during both the pre- and post-operative periods. Animals did not receive antibiotic prophylaxis. The experimental study was conducted under semi-sterile conditions.

Rats were randomly divided into three groups: (1) Primary suture group consisting of 15 rats; (2) fibrin sealant group consisting of 15 rats; and (3) control group (which was used only for bleeding time measurements) consisting of 6 rats. Anesthesia was induced with 75 mg/kg ketamin HCl and 5 mg/kg xylazine HCl (Rompun®)

**Table 1 Findings obtained from experimental groups**

| Subgroup | Primary suture | | | FIbrin sealant | | | Control |
|---|---|---|---|---|---|---|---|
| | A | B | C | A | B | C | |
| Adhesion score | 2.50 ± 0.5 | 2.75 ± 0.5 | 1.75 ± 0.5 | 0.25 ± 0.5 ($P < 0.015$) | 0.50 ± 0.6 ($P < 0.06$) | 0.70 ± 0.5 ($P < 0.015$) | - |
| Histopathologic score | 8.75 ± 0.5 | 7.50 ± 1.0 | 6.40 ± 1.7 | 6.75 ± 0.5 ($P < 0.006$) | 5.50 ± 0.6 ($P < 0.021$) | 3.20 ± 1.6 ($P < 0.025$) | - |
| Bleeding time(s) | | 113 ± 78 | | | 59 ± 37 ([a]$P < 0.001$) | | 125.7 ± 74 |

A: Postoperative d 3; B: Postoperative d 10; C: Postoperative d 20. [a]Mann-Whitney *U* test, according to the control group.

im. Then the abdominal wall was shaved off all the hair and the skin was cleaned using Betadine® solution. The abdomen was then entered with a 3-cm median incision to resect a triangular piece with a side length of 1 cm from the inferior edge of the left liver lobe (approximately 4% of the overall liver weight). In the primary suture group, horizontal matrix sutures (6/0 polypropylene) were applied to repair the liver injury. In the fibrin sealant group, the liver surface was coated with fibrin sealant (Tissel Kit®, 2.0 mL, Baxter AG). The fibrin sealant kit contains proteins and thrombin, properly cooled and dried, as well as a $CaCl_2$ and aprotinin solution. Mixing these substances results in two components: a covering and a thrombin solution. After the administration of primary suture and fibrin sealant, both test groups underwent chronometric measurements to determine the bleeding times. Blood samples (1 mL) were taken from the inferior vena cava to detect AST, ALT and ALP values. Five rats from each of the two test groups were sacrificed by administration of ether anesthesia at high doses on 3rd, 10th and 20th postoperative days. Laparotomy was performed by an U-shaped incision curved to upwards. Development of intra-abdominal adhesions was then assessed in animals for qualitative aspects, which were defined formerly as the following phases: Phase 1: Avascular, transparent, thin adhesion; Phase 2: Partly vascular, medium thick adhesion distinguishable with blunt dissection; Phase 3: Vascular, barely thick adhesion distinguishable with sharp dissection[10]. Samples were taken from the recovering liver region.

Light microscopy was employed for the evaluation and scoring of hepatic regeneration based on the following criteria: 1 = necrosis, 2 = hemorrhage, 3 = cytoplasmic vacuolization, 4 = multinuclear large cells, 5 = fibro-vascular structures, 6 = inflammatory exudates. Scores ranging from 0 to 3 were interpreted as the following: 0 = absence of any of these parameters, 1 = slight levels, 2 = medium levels, and 3 = high levels attained by values for the same, which were processed for calculating the total histopathologic regeneration score[11]. Points calculated at high levels indicated that the regeneration had an immature character. Total histopathologic regeneration scores were calculated for each group. AST, ALT and ALP values were assessed as the liver enzymes indicating cellular damage.

Mann-Whitney *U* and Chi-square tests were used for statistical analyses when appropriate.

## RESULTS

The mean bleeding times were 125.7 ± 74 s, 113 ± 78 s and 59 ± 37 s in the control group, primary suture group and fibrin sealant group, respectively, indicating that the bleeding time was significantly shorter in the fibrin sealant group as compared to the control group and primary suture group ($P = 0.001$). However, no significant difference ($P = 0.069$) in bleeding time was found between the control group and primary suture group. Results of the test groups are shown in Table 1.

In the primary suture group, tearing on the liver surface and bleeding were observed during the suturing procedure; abscess formation was noted on suture lines over the liver in one rat on d 3, in an another rat on d 10 and in two other rats on d 20 postoperatively. At the 10th and 20th postoperative days, wound regions could not be observed clearly due to intensive adhesions between the liver and the great omentum.

A second administration of fibrin sealant was required in two rats of the fibrin sealant group due to the separation of the wound surfaces on d 3. In this group, the sites of hepatic resection were proven to be indistinguishable from the normal parenchyma on the 20th postoperative day; and there was no abscess development in the any rat liver throughout the postoperative study period; few adhesions were observed between the liver and the omentum. Although the AST, ALT and ALP levels were found to be lower in fibrin sealant group on the 3rd, 10th and 20th as compared to the primary suture group, but the difference could not reach statistical significance.

Significantly more intra-abdominal adhesions were observed in the primary suture group compared to the fibrin sealant group on 3rd ($P = 0.015$), 10th ($P = 0.006$) and 20th ($P = 0.015$) postoperative days.

The mean histopathological regeneration scores indicated a significantly more wound immaturity in the primary suture group than in the fibrin sealant group on the 3rd ($P = 0.006$), 10th ($P = 0.021$) and 20th ($P = 0.025$) postoperative days. In the liver regeneration areas of necrosis and hemorrhage appeared to be less in the fibrin sealant group on the 3rd postoperative day ($P = 0.014$ and $P = 0.003$, respectively) as compared to the primary suture group. Cytoplasmic vacuolization was found to be significantly higher in primary suture group on the 20th postoperative day ($P = 0.014$) as compared to the primary suture group.

## DISCUSSION

Deep hepatic sutures are known to prove inefficient in

stopping bleeding from the portal veins and branches of hepatic arteries as well as the posterior hepatic vein[12]. Recent studies tend to concentrate on investigating the risk of formation of intra-hepatic hematoma and abscess as well as the development of areas of necrosis dependent on parenchymal ischemia[12]. In the present study, the parenchymal suture group required a significantly longer duration for hemostasis (113 ± 78 s) than the fibrin sealant group (59 ± 37 s). We prefer using polypropylene sutures, a synthetic non-absorbable material known to have a more inert composition and pose lesser risk for infections. In some previous studies, it has been demonstrated that the thrombus generated after the administration of fibrin sealant may have less potential for the occurrence of the infection[13].

In the present study, the fibrin sealant provided shorter hemostasis times following the application. It was hard to re-find the operation area in fibrin sealant administered rats even on the 3rd postoperative day. In two rats, however, an extra fibrin sealant application was required to ensure the merging of the two surfaces, yet neither of these rats indicated any signs of secondary bleeding on surfaces of injured livers. Jacob *et al*[14] have recently reported that fibrin sealants are beneficial even in cases with prolonged hemostasis in rats with liver injuries. They demonstrated that fibrin impregnated collagens-enhanced survival compared to primary sutures, fibrin sealant was absorbed completely on d 28. In another study[15] performed on dogs, an efficient hemostasis was obtained using fibrin sealant in both superficial and profound injuries, no signs of hematoma or secondary bleeding were encountered and the fibrin sealant was completely absorbed within 6 wk of application. Chonn *et al*[16] administered the fibrin sealant in addition to performing surgery using standard surgical techniques subsequent to a liver injury created by infliction of an external blast effect. However, most of subjects in the control group required perihepatic packing, in the fibrin sealant administered group none of the subjects revealed a need for perihepatic packing and re-laparatomy[16]. Similarly, another study demonstrated that fibrin glue eliminated the need for packing after severe liver injuries[17]. Holcomb *et al*[18] attempted to apply the dry fibrin sealing dressing composed of concentrated fibrinogen, thrombin and calcium on polyglyconate mesh on pigs with grade V experimental liver injury. They consequently reported that dry fibrin sealing dressing provided a simple but quick and efficient control over hemorrhage without change of efficacy with neither hypothermia nor coagulopathy[18]. Moreover, several experimental studies have advocated the effectiveness of the novel fibrin sealant[19-21].

In this study, the highest incidence of abscess formation (33%) was observed in the primary suture group postoperatively, while no abscess was observed in the fibrin sealant group. This can be explained by the features of this technique which does not allow occurrence of any ischemic or blind areas or hematoma formation, and the fibrin sealant generates a quick and permanent sealing on blood and lymphatic veins by stopping fibrin exudation. According to the Dulchavsky's study, autologous fibrin gel possesses bactericidal properties in contaminated hepatic injuries[22]. Likewise, in the Taha's experimental study, the number of occurrences of abscesses was less extensive in the fibrin adhesive group than the suture group[23].

Dulchavsky's study with fibrin gel has demonstrated a significant improvement in adhesion formation and intra-abdominal abscess rate as compared with suture hepatorrhaphy[22]. The grade of adhesions found between the liver and omentum was observed to be drastically lower in the fibrin sealant group, the reason of which was attributed to minimal tissue damage and non-existent ischemia in liver parenchyma ensured with this technique[22].

The results obtained in this study are in compliance with those of past studies showing completion of liver, spleen and renal regeneration on d 30 following fibrin sealant administration[11]. Wound healing appeared to be completed at a high extent based on the findings of macroscopic evaluations in this group on d 20. The histological findings obtained also showed that hepatic healing was at higher rates in the fibrin sealant group than in the other groups. Tovar *et al*[11] investigated the effects of fibrin sealant administration on hemostasis and hepatic healing after hepatectomy. Fibrin sealant administration technique provided a faster hemostasis, while the hepatic recovery in the same group revealed to be quicker compared to hot air coagulation and primary suture techniques[11]. The results of this study are in agreement with those obtained in our experimental study. Previous studies demonstrated that plasminogen activators had an important additive role in liver regeneration by their contribution on remodelling of the liver[24,25]. Contrary to the presence of aprotinin in the fibrin sealant kit which is known as an plasminogen inhibitor, no negative effect on hepatic healing was observed. Vice versa, hepatic healing was more rapid than the other group. We believe that further studies need to be performed on this particular issue.

Kohno *et al*[26] compared the efficiencies of microcrystal collagen dust versus the fibrin sealant following an elective hepatic resection in 62 patients. They encountered bile leakage in two patients, and secondary hemorrhage in one patient in the collagen group, while no such complications were observed in fibrin sealant administered group[26]. Even though the study of Figueras *et al*[27] could not justify the influence of fibrin sealant, many clinical studies are available in the literature showing the useful effects of fibrin sealant in prevention of bile leakage after hepatic resection[27-31].

In conclusion, fibrin sealant may be preferred over to primary suture in appropriate cases of liver trauma, due to the shorter bleeding time, faster regeneration and lesser intra-abdominal adhesions by its use. However, more studies focusing on the clarification of the effects of this product on liver regeneration are needed to check the validity of these results.

## COMMENTS

### *Background*

Liver injuries occur as a result of blunt and penetrating traumas and rarely due to some iatrogenic reasons. In blunt abdominal traumas, liver turns out to be the organ that mostly gets injured. Acute bleedings and operative complications cause an increase in rate of mortality and morbidity.

### *Research frontiers*

Fibrin sealant can be used as a safe and appropriate treatment technique in

liver injuries. There were many clinical and experimental studies in the literature showing the useful effects of fibrin sealant in prevention of bile leakage and in shortening bleeding time after hepatic resection and liver injury. However, the effects on liver regeneration and development of intra-abdominal adhesions yet remain unclear.

### Innovations and breakthroughs

In this experimental study, more intra-abdominal adhesions were observed in the primary suture group compared to the fibrin sealant group. Hepatic healing was faster and liver abscess was not observed in the fibrin sealant group postoperatively. Minimal tissue damage and non-existent ischemia in liver parenchyma ensured with application of this technique.

### Applications

Fibrin sealant can be used clinically in appropriate cases including liver trauma or hepatic resection.

### Terminology

The fibrin sealant kit contains proteins and thrombin, properly cooled and dried, as well as a calcium chloride and aprotinin solution. Mixing these substances and covering the tissue surface result a thrombin solution.

### Peer review

In this experimental study, the authors revealed that fibrin sealant causes less intra-abdominal adhesions and faster hepatic healing while allowing shorter hemostasis time compared to the primary suture as assessed in experimental liver trauma. Therefore, fibrin sealant can be preferred over primary suture in appropriate cases, including liver trauma.

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*RAPID COMMUNICATION*

# C-reactive protein levels during a relapse of Crohn's disease are associated with the clinical course of the disease

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## Abstract

**AIM:** To explore if C-reactive protein (CRP) levels might serve as a prognostic factor with respect to the clinical course of Crohn's disease and might be useful for classification.

**METHODS:** In this retrospective cohort study we enrolled 94 patients from the inflammatory bowel disease (IBD) database of the University Medical Centre Utrecht. CRP levels during relapse were correlated with the number of relapses per year. Severity of relapses was based on endoscopic reports and prednisone use. Furthermore, patients were categorized in a low or high CRP group based on their CRP response during relapse and demographic and clinical features were compared.

**RESULTS:** Overall, a positive correlation between CRP levels, number of relapses, and severity of relapse was found (respectively $r_s$ = 0.31, $P$ < 0.01 and $r_s$ = 0.50, $P$ < 0.001). Employing a cut-off level of 15 mg/L, the index CRP level was found to discriminate patients with respect to the number of relapses per year, as well as for severity of relapses (respectively 0.25 ± 0.16 *vs* 0.36 ± 0.24, $P$ < 0.05 and 4.4 ± 1.2 *vs* 3.2 ± 1.1 on a 10-point visual analogue scale, $P$ < 0.001 for the high CRP and low CRP groups respectively). In addition, the high CRP group showed more cumulative days of prednisone use per year (107 ± 95 *vs* 58 ± 48, $P$ < 0.05), as well as a better response to infliximab (93 % *vs* 33 %, $P$ = 0.06).

**CONCLUSION:** A higher CRP level during relapse seems to be associated with a more severe clinical course of disease.



**Key words:** Crohn's disease; Infliximab; C-reactive protein; Prognosis; Clinical behaviour

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## INTRODUCTION

Crohn's disease (CD) is thought to result from an ongoing activation of the mucosal immune system leading to an inappropriate innate immune response to normal luminal factors in a genetically susceptible individual[1,2]. The clinical expression of CD is heterogeneous with a wide spectrum of patterns and different clinical courses. Classification of CD is, therefore, difficult[3]. In the Vienna Classification, distinct definitions to categorize patients with CD have been formulated[4]. This classification distinguishes between age (< or > 40 years), anatomical localization (terminal ileum, colon, ileocolon, upper gastrointestinal) and disease behaviour (non-stricturing/non-penetrating, stricturing and penetrating). However, it has been shown to be unreliable in predicting the course of the disease[5,6]. Currently, it is not known whether other tools, such as the recently developed Montreal classification will prove to be superior in this respect[7,8].

C-reactive protein (CRP), an acute phaseprotein produced by hepatocytes upon activation by proinflammatory cytokines such as IL-6, IL-1α and tumour necrosis factor-alpha (TNF-α) is widely used as a parameter of inflammatory activity in a variety of infectious and inflammatory diseases[9-11]. Moreover, it has been found to correlate with clinical parameters of disease activity in CD[12-18].

We hypothesized that a high serum CRP level during a relapse predisposes patients to a more severe course of the disease. In the present study, we studied retrospectively the putative association between the height of CRP levels during a relapse and the clinical course of disease in patients with CD.

## MATERIALS AND METHODS

In this retrospective cohort study, 94 patients with CD were selected from the IBD database of the University Medical Centre Utrecht, comprising the records of all patients diagnosed with IBD in the period 1994-2005.

CD patients were eligible if they were 18 years of age or older, had experienced at least one well-documented relapse during which serum CRP values were determined, and from whom records over a period of at least 4 years of follow-up were available. Excluded were patients with other chronic inflammatory diseases, concomitant infections or other serious comorbidity.

Data were collected from electronic and clinical charts. The index CRP level used for analysis had to be measured during a relapse which was confirmed by (ileo) colonoscopy and/or a small bowel follow-through examination. The highest CRP level during this exacerbation, within the first 4 wk after the onset of symptoms, was taken as an index CRP level. Severity of the index exacerbation was assessed retrospectively by two independent, blinded reviewers based on the endoscopic reports and recorded on a 10-point visual analogue scale (VAS). The mean of both scores was used for analysis.

The total number of relapses during follow up was assessed. These relapses were defined as a period of inflammatory activity accompanied by progressive symptomatology which was confirmed by (ileo) colonoscopy and/or a small bowel follow-through examination and requiring hospitalization and/or initiation of treatment with steroids or other immunosuppressive drugs. Another primary endpoint was the use of prednisone, measured in number of days during follow up.

Furthermore, age at the time of the diagnosis of CD, duration of the disease, localization and behaviour of CD according to the Vienna Classification, the presence of granulomas in pathology specimens, smoking behaviour, surgical treatment and treatment with immunosuppressives other than prednisone (azathioprine, mesalazine, methotrexate, budesonide, cortisone enema's or infliximab) were noted.

### Statistical analysis

Analysis was performed using SPSS for Windows, version 12.0. The correlation between the index CRP value and the primary outcome measures were calculated employing a bivariate correlation with a non-parametric distribution (Spearman's rho). Differences in the primary, as well as the secondary outcomes of the Low C-reactive protein subgroup (LCRP) and High C-reactive protein subgroup (HCRP) groups were calculated with the Mann Whitney *U* test to compare means (non-parametric distribution) and the chi-square test to compare the differences in distribution (non-parametric distribution).

All tests were 2-tailed analysed. Data are presented in mean ± SD or otherwise stated. A *P*-value of $< 0.05$ was considered statistically significant.

## RESULTS

### Correlation variables

For the whole group, a significant positive correlation between the index CRP level and the number of relapses per year was noted ($r_s = 0.31$, $P < 0.01$, Figure 1). Severity of relapses was correlated with the index CRP level as well ($r_s = 0.50$, $P < 0.001$). The severity was based on the mean score of both independent reviewers with an interobserver correlation of $r_s = 0.71$, $P < 0.001$. The correlation between the index CRP level and cumulative use of prednisone per year was small but significant, $r_s = 0.22$, $P < 0.05$.

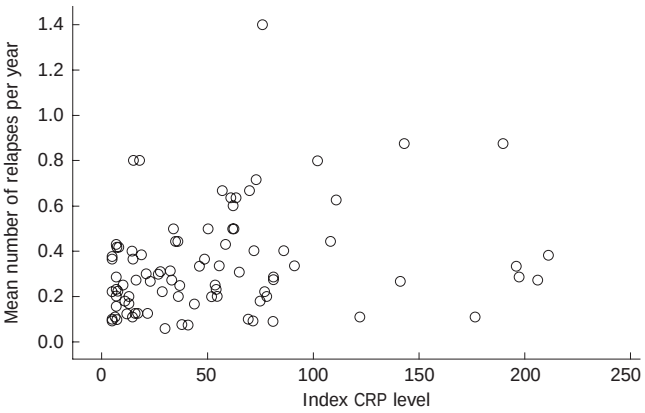


**Figure 1** Correlation between the index CRP and the mean number of relapses per year. Spermann's rho = 0.31, $P < 0.01$.

### Comparison of subgroups

Based on the index CRP levels two groups were created, the LCRP and HCRP group, with a cut-off level of 15 mg/L. Twenty-seven patients had a serum CRP level of ≤ 15 mg/L and 67 had a CRP level of > 15 mg/L. Baseline characteristics of the subgroups are given in Table 1. The baseline characteristics of the groups were similar. There were no differences between the two groups regarding gender distribution, mean age, age at the time of diagnosis, disease localization and disease behaviour according to the Vienna classification.

Table 2 shows the clinical findings, drug use, surgeries and related factors in the two groups. The numbers of relapses were respectively 0.25 ± 0.16 and 0.36 ± 0.24 per year in the LCRP and HCRP group ($P < 0.05$). The mean severity of the index exacerbation on a 10-point VAS was 4.4 ± 1.2 in the HCRP and 3.2 ± 1.1 in the LCRP group ($P < 0.001$). The number of courses of prednisone did not differ between the groups, but patients in the HCRP group were treated significantly longer with prednisone than LCRP-patients, 107 ± 95 *vs* 58 ± 48 d per year ($P < 0.05$).

There was no significant difference in the number of patients on infliximab in both groups, 20.9 % in the HCRP and 11.1% in the LCRP group, but the response rate was higher in the HCRP subgroup compared to the LCRP subgroup, respectively 92.8% *vs* 33.3%, $P = 0.06$.

In the follow-up of patients in the HCRP and LCRP groups, the percentages of azathioprine (77.6 *vs* 66.7), mesalazine (98.5 *vs* 100), budesonide (71.6 *vs* 63.0), cortisone enema's (43.3 *vs* 44.4) and methotrexate (4.5 *vs* 3.7) use did not differ significantly, nor did the total number of CD-related surgeries [1.7 (SD 0.1) *vs* 2.0 (SD 0.6) per patient], construction of stomas (22.4 % *vs* 22.2 %) or development of fistulas and/or abscesses (26.9 % *vs* 18.5%).

There were no significant differences between smokers and non-smokers. Patients with granulomas were significantly younger (38.4 ± 8.5 *vs* 45.4 ± 13.3 years, $P < 0.05$) and tended to be younger at the time

**Table 1 Baseline characteristics of patients with an index serum C-reactive protein (CRP) level ≤ 15 and > 15 mg/L**

| Index serum CRP level during relapse (mg/L) | ≤ 15 | > 15 | *P*-value |
|---|---|---|---|
| | LCRP | HCRP | |
| *n* | 27 | 67 | |
| Time of follow-up, yr (SD) | 10.8 (4.0) | 10.3 (3.7) | NS |
| Demographic factors | | | |
| Sex: men | 12 (44.4%) | 32 (47.7%) | NS |
| Mean age, yr (SD) | 45 (13.5) | 42 (11.0) | NS |
| Clinical features | | | |
| Age at time of diagnosis (SD) | 27.0 (8.3) | 27.4 (10.8) | NS |
| Duration of disease, yr (SD) | 18.0 (10.2) | 14.3 (7.3) | NS |
| Localization | | | |
| Terminal ileum | 6 (22.2%) | 8 (11.9%) | NS |
| Colon | 7 ( 25.9%) | 31 (46.2%) | |
| Ileocolon | 13 (48.1%) | 26 (38.8%) | |
| Upper gastrointestinal | 1 (3.7%) | 2 (3.0%) | |
| Behaviour | | | |
| Non-stricturing non penetrating | 13 (48.1%) | 26 (38.8%) | NS |
| Stricturing | 7 (25.9%) | 15 (22.4%) | |
| Penetrating | 7 (25.9%) | 26 (38.8%) | |

Data are given in numbers and percentages, unless shown otherwise. Significance between the two groups is calculated. Localization and behaviour according to the Vienna Classification of Crohn's disease. HCRP: High CRP response; LCRP: Low CRP response; NS: No significance; SD: Standard deviation.

**Table 2 Subgroups with an index serum C-reactive protein (CRP) level of ≤ 15 and > 15 mg/L, comparison of clinical findings, drug use, surgeries and related factors**

| Index serum CRP level during relapse (mg/L) | ≤ 15 | > 15 | *P*-value |
|---|---|---|---|
| | LCRP | HCRP | |
| Clinical findings | | | |
| Mean number of relapses per year (SD) | 0.25 (0.16) | 0.36 (0.24) | < 0.05 |
| Mean severity of the index relapse according to endoscopic reports, on a 10 points visual analogue scale (SD) | 3.18 (1.09) | 4.39 (1.24) | < 0.001 |
| Drug use | | | |
| Number of patients with prednisone use | 23 (85.2%) | 62 (92.5%) | NS |
| Mean number of days of prednisone use per year (SD)[1] | 58 (48) | 107 (95) | < 0.05 |
| Surgeries | | | |
| Number of patients who underwent ≥ 1 surgeries | 12 (44.4%) | 27 (37.3) | NS |
| Total number of surgeries[2,3] | 2.0 (0.6) | 1.7 (0.09) | NS |
| Related factors | | | |
| Smoking: *n* | 23 | 58 | |
| smokers | 8 (34.8%) | 24 (41.4%) | NS |
| non-smokers | 11 (47.8%) | 24 (41.4%) | |
| quitted smokers | 4 (17.4%) | 10 (17.2%) | |
| Presence of granulomas: *n* | 22 | 51 | |
| | 8 (36.4%) | 25 (49.0%) | NS |

[1]Number of days are based on the mean number of months per year; [2]Calculated mean is based on these patients who underwent surgery; [3]Total of ileocecalresection, partial ileum resection, partial colon resection, (sub) total colectomy and proctectomy. HCRP: High CRP response; LCRP: Low CRP response; NS: No significance; SD: Standard deviation. Data are given in number and percentage, unless shown otherwise.

of diagnosis (24.2 ± 5.9 *vs* 29.7 ± 12.1 years, $P = 0.09$) compared with patients without granulomas. Other differences were not detected.

## DISCUSSION

We hypothesized that the CRP response during a relapse of CD discriminates between phenotypic subsets of patients with an aggressive or more benign course of disease and predicts the response to drugs. We found a significant but relatively modest correlation between level of CRP during the index exacerbation and the number, as well as the severity of relapses per year. A significant, but rather small, correlation between CRP level and prednisone use was revealed. LCRP and HCRP groups were found to be comparable regarding baseline characteristics but the number and severity of relapses was significantly higher in HCRP patients. Furthermore, patients in the HCRP group appeared to respond better to infliximab infusions. The latter phenomenon is in line with literature. This apparently more aggressive disease behaviour in the HCRP patients was not reflected in a higher frequency of drug use other than prednisone. A possible explanation is the tertiary referral setting of this study, resulting in enrolment of relative severe cases of CD in most of whom immunomodulators were already prescribed.

Interestingly, we found the index CRP and severity of relapse based on endoscopic reports to be correlated. CRP levels are found to be weakly associated with clinical activity indices by several authors[14,19-21], although this has not been reported consistently[22]. Colombel *et al* reported a striking correlation of CRP levels with radiologic findings of perienteric inflammation[12]. Whether involvement of mesenteric adipocytes in the immune response results in an increased CRP level and, in the long run, the course of disease, is a matter of speculation. We could not confirm the data from Floren *et al*, who reported an exclusive ileal disease distribution in a group of CD patients with a CRP level below the level of 10 mg/L. In this study the LCRP group had a significant lower body mass index as well, while no differences were found in the frequency and distribution of CARD15 variants[23]. The association of colonic disease phenotype, cigarette smoking or the presence of granulomas with relapses as reported in other studies was not reproduced in the current study either[24-30].

Our study has its limitations. The severity of index relapses was scored retrospectively and the presence of relapses during the follow-up period was assumed if certain criteria were met in the charts. We tried to overcome these limitations by using blinded assessment of the index colonoscopies by two gastroenterologists and by defining strict criteria for relapse in the follow-up phase. The number of courses of prednisone had to be scored retrospectively as well, which only gives a global impression. In addition, we had to choose a cut-off level of CRP to create two groups. In literature, there is only one comparable study[23] in which 10 mg/L was used as the cut-off level. We found that a CRP level of 15 mg/L discriminated the two groups optimally.

In conclusion, this study showed that the level of CRP

during a relapse of CD can serve as a prognostic factor for the number and severity of relapses. Patients with a CRP level > 15 mg/L experienced more and more severe relapses, were treated more extensively with prednisone and responded better to infliximab therapy. However, we consider these differences too small to be of use in clinical decision making.

## COMMENTS

### Background

Crohn's disease (CD) may affect any part of the gastrointestinal tract and is characterized by a broad spectrum of clinical presentations and a variable disease course. Disease flares occur randomly and cannot be predicted reliably. The aim of the present study was to explore the value of C-reactive protein (CRP) as a prognostic factor with respect to the clinical course of CD.

### Research frontiers

Early identification of CD patients with a propensity to an aggressive or more benign disease course could be of major clinical importance; this might guide the clinician in deciding what maintenance therapy should be prescribed and could even result in early initiation of aggressive induction therapy to avoid flares or complications. To date, a simple parameter, reliably predicting disease activity and flares is not available. From all laboratory markers, CRP seems to be the most promising candidate in this respect. Not all patients with confirmed disease activity, however, display raised CRP levels. We hypothesized that this group constitutes a distinct, benign phenotype, possibly requiring less intensive treatment.

### Innovations and breakthroughs

A high CRP level during relapse is positively correlated with the severity and frequency of relapses. Furthermore, this was found to be associated with longer courses of steroids and a better response following infliximab administration.

### Applications

These findings suggest that phenotypes can be identified using a biochemical parameter such as CRP, which predicts to a certain extent the course of disease and response to drugs. The differences found in this study, however, are too small to guide clinical decision making and the use of CRP in this setting should be seen as an additive tool.

### Terminology

High CRP and low CRP responders are patients that respectively react with a high CRP level and a low CRP level during a relapse of Crohn's Disease.

### Peer review

This is an important contribution to the literature of inflammatory bowel disease. The study is well conceived and the report is well written.

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*RAPID COMMUNICATION*

# Are there tumor suppressor genes on chromosome 4p in sporadic colorectal carcinoma?

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## Abstract

**AIM:** To study the candidate tumor suppressor genes (TSG) on chromosome 4p by detecting the high frequency of loss of heterozygosity (LOH) in sporadic colorectal carcinoma in Chinese patients.

**METHODS:** Seven fluorescent labeled polymorphic microsatellite markers were analyzed in 83 cases of colorectal carcinoma and matched normal tissue DNA by PCR. PCR products were eletrophoresed on an ABI 377 DNA sequencer. Genescan 3.7 and Genotype 3.7 software were used for LOH scanning and analysis. The same procedure was performed by the other six microsatellite markers spanning D4S3013 locus to make further detailed deletion mapping. Comparison between LOH frequency and clinicopathological factors was performed by $\chi^2$ test.

**RESULTS:** Data were collected from all informative loci. The average LOH frequency on 4p was 24.25%, and 42.3% and 35.62% on D4S405 and D4S3013 locus, respectively. Adjacent markers of D4S3013 displayed a low LOH frequency (< 30%) by detailed deletion mapping. Significant opposite difference was observed between LOH frequency and tumor diameter on D4S412 and D4S1546 locus (0% *vs* 16.67%, $P$ = 0.041; 54.55% *vs* 11.11%, $P$ = 0.034, respectively). On D4S403 locus, LOH was significantly associated with tumor gross pattern (11.11%, 0, 33.33%, $P$ = 0.030). No relationship was detected on other loci compared with clinicopathological features.

**CONCLUSION:** By deletion mapping, two obvious high frequency LOH regions spanning D4S3013 (4p15.2) and D4S405 (4p14) locus are detected. Candidate TSG, which is involved in carcinogenesis and progression of sporadic colorectal carcinoma on chromosome 4p, may be located between D4S3017 and D4S2933 (about 1.7 cm).



**Key words:** Loss of heterozygosity; Colorectal carcinoma; Chromosome 4p; Tumor suppressor gene

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## INTRODUCTION

Colorectal cancer (CRC) constitutes the second most common neoplasm in Western countries and is the third leading cause of cancer-related death, the overall 5-year survival rate is approximately 45%[1]. Improvement in its prognosis can not be achieved without a better understanding of its etiology and tumor molecular biology. In recent years, the genetic basis of human tumors has been increasingly elucidated. As a model for both multistep and multipathway carcinogenesis, colorectal neoplastic progression provide paradigms of both oncogenes and tumor suppressor gene in epithelial tumors[2,3]. The latter changes predominate. In addition to the allelic loss on chromosome 5q, 17p and 18q, many other chromosome losses can be observed in colorectal carcinoma. Regions on chromosome 1q, 4p, 6p, 6q, 8p, 9p and 22q were lost in 25%-50% of the colorectal tumor cases studied previously[2].

Chromosome losses in colorectal tumor were first detected by cytogenesis, later, by probes of restriction fragment length polymorphisms (RFLP) and now by loss of heterozygosity (LOH) in analyzing allelic loss. The loss of tumor suppressor genes is believed to be one of the key steps to carcinogenesis of colorectal cancer[4]. The loss of one allelic at specific locus is caused by deletion mutation or loss of a chromosome from a chromosome pair[5]. When this occurs at a tumor suppressor gene locus where one of the allelics is already abnormal, it can result

in neoplastic transformation. The LOH analysis based on polymorphic microsatellite DNA has become an effective and powerful tool currently to find informative loci and candidate tumor suppressor genes[6,7]. Most investigations concentrated on defining the minimal regions of loss of specific chromosomes in various cancers in an effort to identify the putative tumor suppressor genes targeted by the loss[8].

In this study, we first analyzed the LOH events on chromosome 4p using seven microsatellite markers and made further refined deletion mapping analysis spanning D4S3013 locus in 83 sporadic colorectal carcinoma cases in an attempt to identify additional candidate tumor suppressor genes involved in colorectal tumorigenesis.

## MATERIALS AND METHODS

### *Patient sample and DNA extraction*

This study was based on consecutively collected tumors in 83 patients with colorectal cancer, including 40 males and 43 females, treated at the surgical department in Shanghai First People's Hospital, China. The patients' ages ranged from 31 to 84 years with a median of 66. The cancerous tissue and adjacent normal control tissue (> 10 cm) were freshly frozen. The tissues were cut into cubes of approximately 2 $mm^3$ and immediately frozen in liquid nitrogen. Each patient gave his or her informed consent for the use of his or her tissue in this study. DNA was extracted using standard methods with proteinase K digestion and phenol/chloroform purification[9]. All patients were confirmed by pathology and were staged by Duke's criteria.

### *Microsatellite markers and PCR*

Initially, 83 cases of colorectal cancer were analyzed by PCR using seven microsatellite markers (Shanghai Biology Technology Company, China) which map to chromosome 4p. DNA samples were analyzed as matched normal and tumor pairs using primers of the following microsatellite loci (hereditary location/heterozygote ): pter-D4S412 (4p16.3/76)-D4S2935 (4p16.1/62)-D4S1599 (4p16.1/81)-D4S303 (4p15.33/76)-D4S3013 (4p15.2/84)-D4S391 (4p15.2/85)-D4S405 (4p14/85). The average hereditary distance was 8.65 cm[10] (Figure 1A). As the D4S3013 locus showed high LOH frequency (35.62%), six additional microsatellite markers map to chromosome 4p15 were employed to further investigate LOH. The same DNA samples were then analyzed as matched pairs for the following microsatellite markers (location/heterozygote): pter-D4S2926 (4p15.32/80)-D4S1546 (4p 15.31 /77) -D4S3017 (4p 15.31/82)-D4S2933 (4p 15.31/60)-D4S2948 (4p 15.2 /81)-D4S1551 (4p 15.2/78). The average hereditary distance was restricted within 1.03 cm[10] (Figure 1B).

### *LOH result analysis*

A portion of each PCR product (0.5 μL) was combined with 0.1 μL Genescan 500 size standard (PE Applied Biosystems Foster City, CA, USA) and 0.9 μL formamide loading buffer. After denaturation at 96℃ for 5 min, products were eletrophoresed on 5% polyacrylamide

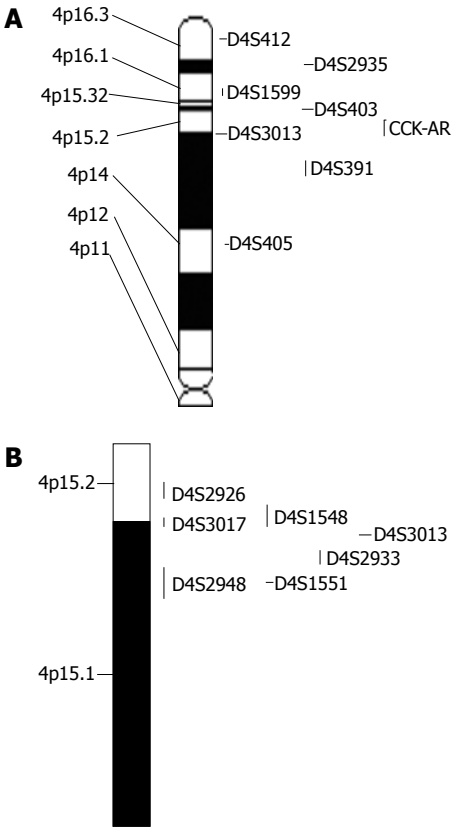


**Figure 1** **A:** Microsatellite markers and candidate tumor suppressor genes on 4p (www.gdb.org); **B:** Microsatellite markers on D4S3013 (www.gdb.org).

gels using an ABI 377 DNA sequencer (PE Applied Biosystems Foster City, CA, USA) for 2.5 h. Genotype 3.7 software display individual gel lanes as electropherograms with a given size, height, and area for each detected fluorescent peak. Stringent criteria were used to score the samples. Alleles were defined as the two highest peaks within the expected size range. A ratio of T1:T2/N1:N2 of less than 0.67 or greater than 1.50 was scored as a loss of heterozygosity (Figure 2). Most amplified normal DNA produced two PCR products indicating heterozygosity. A single fragment amplified from normal DNA (homozygosity) and those PCR reactions, in which fragments were not clearly amplified, were scored as not informative. The LOH frequency of a locus is equal to the ratio of the number between allelic loss and informative cases. The average LOH frequency of chromosome 4p is the average value of each locus.

### *Statistical analysis*

Comparisons between LOH and clinicopathological data were performed by $\chi^2$ test. $P < 0.05$ was considered as statistically significant.

## RESULTS

### *LOH analysis of colorectal cancer on 4p*

Eighty-three colorectal cancers were analyzed for LOH at the seven marker loci spanning chromosome 4p. All loci got informative messengers. The average LOH frequency on 4p was 24.25%. Sixty-three samples (75.90%) showed

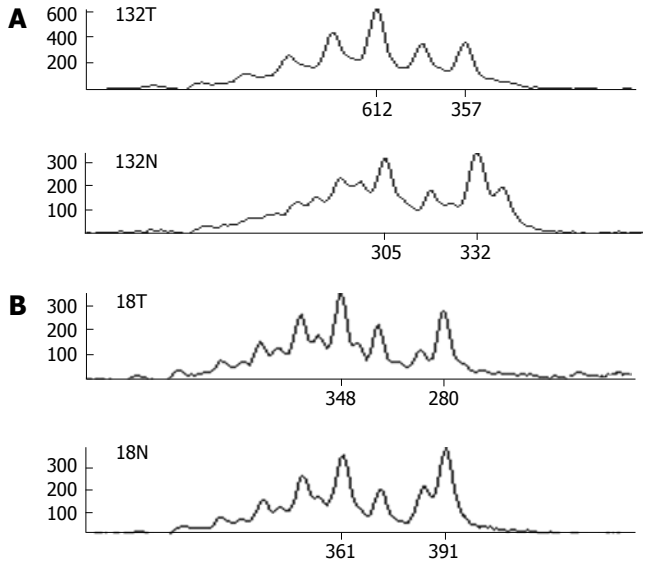


**Figure 2** LOH Demonstration. **A:** Classic LOH peak: Allele ratio = (T1/T2)/(N1/N2) = (612/357)/(305/332) = 1.87 > 1.5; **B:** Heterozygosity retention: Allele ratio = (T1/T2)/(N1/N2) = (348/280)/(361/391) = 0.67 < 1.34 < 1.5. T: Tumor; N: Normal.

**Table 1 LOH result and hereditary distance/location of chromosome 4p**

| Locus | Location | LOH cases | Normal cases | Informative rate | Distance (cM) | LOH rate (%) |
|---|---|---|---|---|---|---|
| D4S412 | 4p16.3 | 5 | 57 | 74.7 | - | 8.06 |
| D4S2935 | 4p16.1 | 24 | 55 | 95.18 | 7.4 | 30.38 |
| D4S1599 | 4p16.1 | 9 | 28 | 44.58 | 10.8 | 24.32 |
| D4S403 | 4p15.33 | 3 | 34 | 44.58 | 5.1 | 8.1 |
| D4S3013 | 4p15.2 | 26 | 47 | 86.9 | 7.7 | 35.62 |
| D4S391 | 4p15.2 | 13 | 46 | 71.84 | 7.4 | 22.03 |
| D4S405 | 4p14 | 22 | 30 | 62.65 | 13.4 | 42.3 |

at least one LOH event. Two distinct regions of frequent allelic loss at D4S3013 (4p15.2) and D4S405 (4p14) locus on chromosome were detected (Table 1). The LOH frequency was 35.62% and 42.3%, respectively. This suggested that putative tumor suppressor genes may be located near D4S3013 and D4S405 loci.

### *LOH deletion mapping results on 4p15 encompassing D4S3013*

The chromosome region spanning D4S3013 locus on 4p15 was investigated using a saturation mapping strategy with another 6 microsatellite markers that are closely located within this region (Figure 2). To detect putative tumor suppressor genes easily, we limited average hereditary distance to 1.03 cm. Forty samples (48.19%) showed at least one LOH event. The average LOH frequency spanning D4S3013 was 24.2% (Table 2). We found that adjacent markers of D4S3013 displayed a low LOH frequency (< 30%), especially on D4S2933 locus, much less information was obtained because of more homozygosity.

### *Relationship between clinicopathological features and LOH on 4p*

On D4S412 locus, none LOH was detected in patients with

**Table 2 LOH result and hereditary distance/location of detailed deletion mapping spanning D4S3013**

| Locus | Location | LOH cases | Normal cases | Informative rate | Distance (cM) | LOH rate (%) |
|---|---|---|---|---|---|---|
| D4S2926 | 4p15.32 | 7 | 38 | 54.22 | - | 15.56 |
| D4S1546 | 4p15.31 | 9 | 38 | 56.63 | 1.6 | 19.15 |
| D4S3017 | 4p15.31 | 13 | 46 | 71.08 | 0.5 | 22.03 |
| D4S3013 | 4p15.2 | 26 | 47 | 87.95 | 1.2 | 35.62 |
| D4S2933 | 4p15.31 | 2 | 14 | 19.28 | 0.5 | 12.5 |
| D4S2948 | 4p15.2 | 15 | 49 | 77.11 | 1.9 | 23.44 |
| D4S1551 | 4p15.2 | 12 | 64 | 91.57 | 0.5 | 21.05 |

tumor larger than 5 cm in diameter (0/27), while in patients with tumor less than 5 cm in diameter, LOH frequency was 14.29% (5/35, $P = 0.041$). On the contrary, on D4S1546 locus, LOH frequency was 35.29% (6/17) in the former; and only 10% (3/30) in the latter locus ($P = 0.030$). Notably, on D4S403 locus, LOH was significantly associated with tumor gross pattern. In tumor of the massive, ulcerative and encroaching pattern, the LOH frequency was 10%, 0%, 33.33%, respectively ($P = 0.030$). No significant relationship was found between clinicopathological features and LOH on other loci (data not shown).

## DISCUSSION

Inactivation of tumor suppressor genes appears to be one of the genetic mechanisms involved in the development of colorectal cancer[11,12]. Deletion of tumor suppressor genes occur frequently in human malignances. Such events can be detected using markers from the region of genome that include a tumor suppressor gene. Allelic deletions detected as LOH have been proved useful for mapping regions of DNA that contains tumor suppressor genes, i.e., LOH at specific chromosomal regions strongly suggests the existence of tumor suppressor genes at the relevant segment.

A great deal of evidence supported the presence of tumor suppressor genes in the short arm of chromosome 4. These include the reversion of the immortal phenotype by chromosome 4 transfer[13] and the frequent occurrence of losses in or near the 4p14-4p16 region in bladder cancer[14]. LOH has been observed at distal 4p in sporadic neuroblastoma with an incidence raging from 20% to 29%[15,16]. Using array comparative genomic hybridization, Hurst *et al*[17] reported the loss frequency of 4p to be 52% in bladder cancer. More importantly, Shirapurkar *et al*[18] observed the loss frequency of > 50% at 4p15.1-4p15.3 in malignant mesothelioma and lung carcinoma. LOH on 4p was 21% and > 30% in differentiated adenocarcinoma of stomach as well[19,20]. Head and neck squamous cell carcinoma, invasive cervical cancer and acinic cell carcinoma also showed a high allelic loss frequency[21-23].

In colorectal tumors, previous alletic typing[24], cytogenetic[25-27] and comparative genomic hybridization[28] studies have reported moderate losses(0%-30%) of chromosome 4. These data have not raised special interest in this chromosome as a candidate to harbor a tumor suppressor gene, therefore, colorectal cancer investigations

have not included a detailed analysis of loss in this chromosome. Choi *et al*[29] reported a LOH frequency of 24%-30% at just several loci on chromosome 4 in colorectal cancer. Later, Arribas *et al*[30,31], used AP-PCR method and suggested chromosome 4p14-4p16 may contain tumor suppressor gene, because LOH frequency on D4S2397 was as high as 35%. These reports indicate that 4p14-4p16 region displayed frequent loss in a couple of cancers, so 4p14-4p16 region is of important value for TSG screening.

D4S3013 locus region, 4p15.2, was concordant with several reports in other tumors before[14,16,20,21]. In this study, we investigated the LOH on 4p in 83 sporadic cases of colorectal cancer. The results showed putative tumor suppressor gene may harbor adjacent to D4S405 and D4S3013 locus. We made further detailed deletion mapping spanning D4S3013 locus, and found that the surrounding markers of D4S3013 displayed a low LOH frequency (< 30%). Therefore, we speculate that the candidate TSG may be located between D4S3017 and D4S2933, about 1.7 cm in hereditary distance.

We found several loci were significantly associated with clinicopathological features. On D4S412 locus, no LOH was detected in patients with tumor larger than 5 cm in diameter, while in patients with tumor less than 5 cm in diameter, the LOH frequency was 14.29% ($P = 0.041$). On the contrary, on D4S1546 locus, the LOH frequency showed opposite phenomenon. On D4S403 locus, LOH was significantly associated with tumor gross pattern. Similarly, Arribas *et al*[31] found solely at the D4S2397 locus was indicative of a shorter disease-free survival ($P = 0.027$). Choi *et al*[29] found 4p loss was significantly associated with early onset of colorectal cancer. The effect of 4p loss on the early-onset disease is unlikely to be the result of tumor aggressiveness, because 4p loss was not found to be correlated with cancer-related death. Nishizuka *et al*[20] found 4p LOH had an essentially similar frequency in early and advanced differentiated adenocarcinoma. The differential behavior of LOH at different markers suggested that distinct mechanisms and/or selection presses participate in the mutational event that affect this chromosomal region during the tumorigenic process.

Regarding allelic loss at 4p, cholecystokinin type A receptor (CCK-AR) gene maps near D4S2397[32,33] (Figure 1). Recent reports have suggested that cholecystokinin receptor may function as a tumor suppressor gene[34,35].

In summary, we investigated LOH on 4p in sporadic colorectal carcinoma in Chinese patients and detected two high deletion regions encompassing D4S3013 (4p15.2) and D4S405 (4p14). Candidate TSG, involved in sporadic colorectal carcinoma on chromosome 4p, may be located between D4S3017 and D4S2933 (about 1.7 cm). Further related gene screening and functional studies may contribute to the identification of the tumor suppressor gene in these regions.

## COMMENTS

### Background

Cancer arises from the accumulation of inherited polymorphism (i.e. SNPs) and mutation and/or sporadic somatic polymorphism (i.e. non-germline polymorphism) in cell cycle, DNA repair, and growth signaling genes. Neoplastic progression is generally characterized by the accumulation of multiple somatic-cell genetic alterations as the tumor progresses to advanced stages. The classic mechanism of tumor suppressor gene inactivation is described by two-hit modes in which one allele is mutated (or promoter hypermethylation or a small intragenic deletion) and the other allele is lost through a number of possible mechanisms, resulting in loss of heterozygosity at multiple loci. Loss of heterozygosity is the most common molecular genetic alteration observed in human cancers. In the model of colorectal tumorigenesis, mutational inactivation of tumor suppressor genes predominates.

### Research frontiers

Most genome-wide scans for loss of heterozygosity (LOH) have been conducted at low resolution with a relatively small number of polymorphic markers. For example, an average of 120 microsatellites have been used to determine the allelotype of multiple different human neoplasms in a series of studies since 1995, and the highest density microsatellite allelotype was about 280 polymorphic markers before the year 2000. SNPs are the most common form of sequence variation in human genome, occurring approximately every 1200 base pairs (bps). High density mapping of genetic losses reveals potential tumor suppressor loci and might be useful for clinical classification of individual tumors. SNP array has been introduced recently for genome-wide screening of chromosome imbalance. Higher density SNP array can effectively detect small regions of chromosomal changes and provide more information regarding the boundaries of loss regions.

### Innovations and breakthroughs

A great deal of evidence supported the presence of tumor suppressor genes in the short arm of chromosome 4. Much less studies have been reported in colorectal cancer. Previous allelotyping analysis of cancer by many groups was used with a relatively low density of markers. By deletion dense markers mapping, we detected two obvious high frequency LOH regions spanning D4S3013 and D4S405 locus in colorectal cancer. Candidate TSG, might be located between D4S3017 and D4S2933 (about 1.7 cm).

### Applications

We used this method to detect some major allelic loss regions in genome-wide scans of LOH in patients with colorectal cancer.

### Terminology

LOH is caused by a variety of genetic mechanisms, including physical deletion of chromosome non-disjunction and mitotic non-disjunction followed by republication of the remaining chromosomes, mitotic recombination and gene conversion. The mechanisms of LOH are remarkably chromosome-specific. Some chromosomes display a complete loss. However, more than half of the losses are associated with a only partial loss of a chromosome rather than a whole chromosome. LOH is also a common form of allelic imbalance and the detection of LOH has been used to identify genomic regions that harbor tumor suppressor genes and to characterize different tumor types, pathological stages and progression.

### Peer review

This is a report that describes the LOH events in sporadic colorectal cancer in Chinese patients, further studies must benefit from this paper. The data presented is clear and concise in the text.

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*RAPID COMMUNICATION*

# A paradox: Insulin inhibits expression and secretion of resistin which induces insulin resistance

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## Abstract

**AIM:** To confirm whether insulin regulates resistin expression and secretion during differentiation of 3T3-L1 preadipocytes and the relationship of resistin with insulin resistance both *in vivo* and *in vitro*.

**METHODS:** Supernatant resistin was measured during differentiation of 3T3-L1 preadipocytes. L6 rat myoblasts and hepatoma cell line H4IIE were used to confirm the cellular function of resistin. Diet-induced obese rats were used as an insulin resistance model to study the relationship of resistin with insulin resistance.

**RESULTS:** Resistin expression and secretion were enhanced during differentiation 3T3-L1 preadipocytes. This cellular differentiation stimulated resistin expression and secretion, but was suppressed by insulin. Resistin also induced insulin resistance in H4IIE hepatocytes and L6 myoblasts. In diet-induced obese rats, serum resistin levels were negatively correlated with insulin sensitivity, but not with serum insulin.

**CONCLUSION:** Insulin can inhibit resistin expression and secretion *in vitro*, but insulin is not a major regulator of resistin *in vivo*. Fat tissue mass affects insulin sensitivity by altering the expression and secretion of resistin.



**Key words:** Resistin; Insulin; Insulin resistance

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## INTRODUCTION

Obesity is a worldwide health problem directly linked to several disease processes such as hypertension and type 2 diabetes mellitus[1]. Adipose tissue is not only an organ for passive energy reserve, but also an active endocrine organ secreting a wide range of hormones and other protein factors called adipokines[1,2]. Among the adipokines, resistin is involved in insulin sensitivity and glucose tolerance[3,4] while others are involved in hemostasis, inflammatory and stress responses, and energy balance[5,6].

Resistin, initially identified in screening for adipocyte-specific transcripts down-regulated by treatment with thiozolidinedione (TZDs), belongs to a novel family of cysteine-rich proteins, each with a unique tissue distribution[3, 4]. In rodents, resistin predominantly expressed in white adipose tissue[4] reduces insulin sensitivity in adipocytes and skeletal muscles by impairing insulin-mediated glucose transport and inducing the expression of suppressor of cytokine signaling 3 (SOCS3)[7-9], and regulates fasting blood glucose by increasing hepatic glucose release[10]. Therefore, resistin might provide a link between obesity and diabetes mellitus.

Initial studies on the regulation of resistin indicate that resistin expression is reduced by fasting and increases rapidly on refeeding[3]. Circulating resistin levels are elevated in genetically obese (ob/ob, db/db) mice, and obese is induced by a high-fat diet[3]. Insulin inhibits resistin mRNA expression in 3T3-L1 preadipocytes[11,12]. However, these data do not support a role of resistin in insulin resistance[11]. If resistin is mainly regulated by insulin, the major function of resistin is to induce insulin resistance, forming an insulin-resistin-insulin sensitivity positive feedback loop that cannot exist *in vivo*.

In the present study, resistin expression and secretion were elevated during 3T3-differentiation of L1

preadipocytes. This cellular differentiation-stimulated resistin expression and secretion were suppressed by insulin. Resistin also induced insulin resistance in H4IIE hepatocytes and L6 myoblasts. In diet-induced obese rats, serum resistin levels were negatively correlated with the insulin sensitivity index (ISI). No negative correlation was found between the levels of fasting serum insulin and resistin, suggesting that insulin is not the major regulator of resistin in rodents.

## MATERIALS AND METHODS

### Cell culture

3T3-L1 preadipocytes were cultured at 37℃ in an atmosphere containing 50 mL/L $CO_2$ and 950 mL/L air. The cells were maintained in growth medium consisting of Dulbecco's modified Eagle's medium (DMEM, Gibco BRL, USA), 45 mmol/L glucose, 10% heat-inactivated fetal bovine serum (FBS, Gibco BRL, USA), 2 mmol/L L-glutamine, and 50 U/mL penicillin and 50 ng/mL streptomycin (Sigma, USA). Induction of adipocytic differentiation of 3T3-L1 cells was performed as described elsewhere[13]. Briefly, 3T3-L1 cells were grown in DMEM supplemented with 10% FBS until confluence. Two days after complete confluence (d 0), cells were cultured in DMEM supplemented with 10% FBS and 0.5 mmol/L 1-methyl-3-isobutylxanthine (Sigma, USA), 0.25 μmol/L dexamethasone (Sigma, USA) and 100 nmol/L insulin (Sigma, USA) for 48 h. From d 2 to 4, the full medium was supplemented with 100 nmol/L insulin only. The cells were then switched back to DMEM containing only 10% FBS for the remaining days. Cultures were replenished every 2 d.

The rat hepatoma cell line H4IIE was cultured at 37℃ in an atmosphere containing 50 mL/L $CO_2$ and 950 mL/L air, and maintained in DMEM containing 1 g/L glucose and 10% FBS. The cells were incubated in serum-free DMEM (1 g/L glucose) overnight before assay. Glucose levels were adjusted to 4.5 g/L and H4IIE cells were treated with resistin (50 ng/mL) (Alexis, USA) for 2 h prior to insulin (100 nmol/L) stimulation for 2 h. Glycogen synthesis was then assayed as previously described[14].

L6 rat myoblasts were maintained in DMEM supplemented with 10% FBS and differentiated into myotubes by exposure to DMEM supplemented with 2% FBS. Myogenic differentiation to myotubes was confirmed morphologically and biochemically as previously described[15].

### Resistin secretion

The supernatants of 3T3-L1 preadipocytes were collected on d 0, 4, 6, and 8 after differentiation and centrifuged to remove cells that might have detached from the culture flasks. The supernatants were kept at -20℃ until assayed for resistin content by enzyme immunoassay (ADL, USA).

### RNA preparation and amplification by RT-PCR

Total RNA was isolated from cultured 3T3-L1 cells using the TRIZOL method (Invitrogen, USA). Single strand cDNA synthesis was performed. In brief, the reverse transcription mixture contained 1 μg total RNA, 0.5 μg of oligo d(T) primer, 4 μL of 5 × RT buffer, 0.5 mmol/L deoxynucleotides, 50 U of RNase inhibitor, and 200 U of reverse transcriptase (Promega, USA) in a total volume of 20 μL, the reaction was carried out at 42℃ for 1 h followed by heat inactivation at 95℃ for 5 min. The number of cycles and reaction temperatures used in the PCR assay were optimized to provide a linear relationship between the amount of input template and the amount of PCR product[16]. The primers used for amplification, together with their specific optimum cycling conditions, were as follows:

Mouse resistin (a 415 bp product): [sense primer: 5'-CAA ACAAGACTTCAACTCCC-3', antisense primer: 5'-ACA CACACCCTTCTCCACTA-3', annealing temperature (TA) 58℃, 33 cycles].

β-actin (a 240 bp product): [sense primer: 5'-TAA AGA CCTCTATGCCAACACAGT-3', antisense primer: 5'-CAC GATGGAGGGGCCGGACTCATC-3' annealing temperature (TA) 57℃, 25 cycles].

### Glycogen detection

H4IIE cells were serum starved overnight in DMEM containing 0.2% FBS prior to resistin and/or insulin treatment in all experiments. Cells were lysed with 30% KOH and the vials were kept at 100℃ for 20 min. After addition of anhydrous ethanol, the vials were centrifuged at 4000 × *g* for 15 min with the supernatants discarded. Distilled water (0.5 mL) and 1 mL of 0.2% anthrone [0.2 g of anthrone in 100 mL of 98% $H_2SO_4$ (g/mL), prepared freshly within 1 h] were added, and the vials were placed into boiling water for 20 min. The optical density at 620 nm of the solution in vials was determined by photometry. This method could detect 1.6 μg of glucose per mL, which is equivalent to 1.44 μg of glycogen per mL[17].

### 2-Deoxyglucose uptake assay

Myotubes were serum starved overnight in DMEM containing 0.2% FBS prior to resistin and/or insulin (10 nmol/L 15 min) treatment in all experiments. Uptake of 2-deoxy-D-[3H] glucose (CIC, China) was assayed for 10 min as previously described[18]. Briefly, the cells were washed with ice-cold phosphate-buffered saline, and then 200 μL NaOH (1 mol/L) was added to each well. Aliquots of the cell lysate were transferred to the scintillation vials for radioactivity counting and the remainder were used for protein assay. Non-specific uptake was determined in the presence of cytochalasin B (10 μmol/L) and subtracted from all values.

### Animals

Forty-eight weaned male Sprague-Dawley rats, supplied by the Animal Center of Jiangsu Province, were acclimated to 22℃ in a 12 h light/12 h dark cycle with free access to a standard chow diet for at least a week before grouping. High energy diet contained 10% milk powder, 10% glucose, 10% egg, 10% oil, and 60% standard feed[19]. Animals received this diet for 7 wk. Insulin sensitivity was defined by a value of ISI {ISI = Ln [1/(fasting plasma insulin*glucose)]}[20].

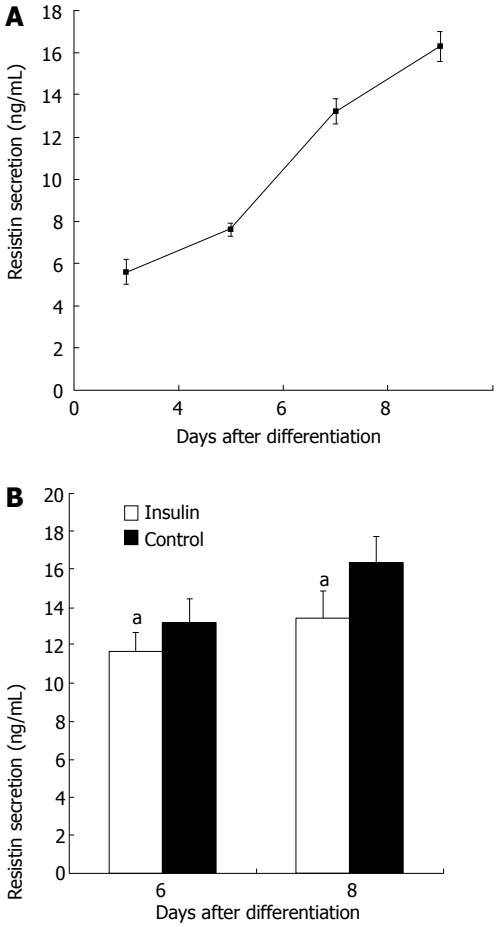


**Figure 1** Increased resistin secretion (**A**) and insulin-inhibited resistin secretion (**B**) during 3T3-L1 preadipocyte differentiation. $^{a}P < 0.05$.

### *Statistical analysis*

The data were presented as mean ± SE. Statistical analysis was undertaken using one-way ANOVA or the paired Student's *t*-test where appropriate. Serum resistin levels in diet-induced obese rats were compared with the insulin sensitivity index and levels using the Bivariate correlation. Differences between groups were considered statistically significant when $P < 0.05$.

## RESULTS

### *Increased resistin secretion during 3T3-L1 preadipocyte differentiation inhibited by insulin*

Resistin secretion was enhanced during 3T3-L1 preadipocyte differentiation ($P < 0.05$, Figure 1A). Resistin secrtion was about 3-fold higher in matured 3T3-L1 adipoctyes (d 8) than in 3T3-L1 preadipocyte (d 0) ($P < 0.05$). The effect of inslulin (100 nmol/L) on resistin secretion was then assessed in cultured 3T3-L1 adipocytes on d 6 and d 8. Six days after induction of differentiation, 100 nmol/L insulin reduced secretion of resistin by 13% ($P < 0.05$, Figure 1B). Eight days after induction of differentiation, 100 nmol/L insulin reduced secretion of resistin by 20% ($P < 0.05$, Figure 1B).

### *Upregulation of resistin mRNA level during 3T3-L1 preadipocyte differentiation inhibited by insulin*

Resistin mRNA was not detectable in undifferentiated 3T3-L1 cells, but was evident by d 4 after the induction of differentiation into adipocytes (Figure 2A). Resistin mRNA was up-regulated during 3T3-L1 preadipocyte differentiation (Figure 2A), and 100 nmol/L insulin decreased resistin mRNA 6 and 8 d after differentiation (Figure 2B).

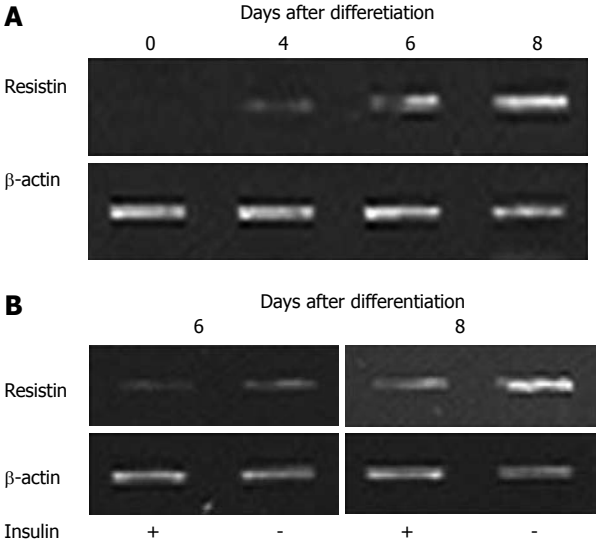


**Figure 2** Up-regulation (**A**) and down-regulation (**B**) of resistin mRNA level by insulin during 3T3-L1 preadipocyte differentiation.

### *Induction of cellular insulin resistance by resistin*

Since insulin could inhibit resistin expression and secretion *in vitro*, additional studies were initiated to determine whether resistin plays a role in insulin resistance. Hepatocytes and myotubes are two important targets of insulin[21]. Glycogen synthesis in insulin-stimulated hepatocytes is the most important marker of hepatocyte insulin sensitivity[22]. After treatment with resistin for 2 h, basal glycogen synthesis decreased about 15% and insulin-stimulated glycogen synthesis decreased about 25% in H4IIE cells ($P < 0.05$, Figure 3A). After treatment with resistin for 2 h, basal 2-deoxyglucose uptake decreased about 50% and insulin-stimulated 2-deoxyglucose uptake decreased about 60% in myotubes ($P < 0.05$, Figure 3B), suggesting that resistin could induce cellular insulin resistance, and both hepatocytes and myotubes are important targets of resistin.

### *Negative correlation of serum resistin with insulin sensitivity but not with serum insulin*

Serum resistin levels correlated with rat ISI ($r = -0.662$, $P = 0.005$) in both control and diet-induced obese rats (Figure 4A). Resistin was positively correlated with insulin ($r = 0.592$, $P = 0.016$, Figure 4B), suggesting that insulin could not inhibit resistin secretion *in vivo*.

## DISCUSSION

Obesity is associated with insulin resistance and type 2 diabetes, implying that adipose tissue plays a role in the development of such disorders[23]. Besides storing fat, adipose tissue is also an active regulation centre, providing signals to guide metabolism by secreting adipokines[1,2]. Resistin is a newly discovered adipokine that is believed

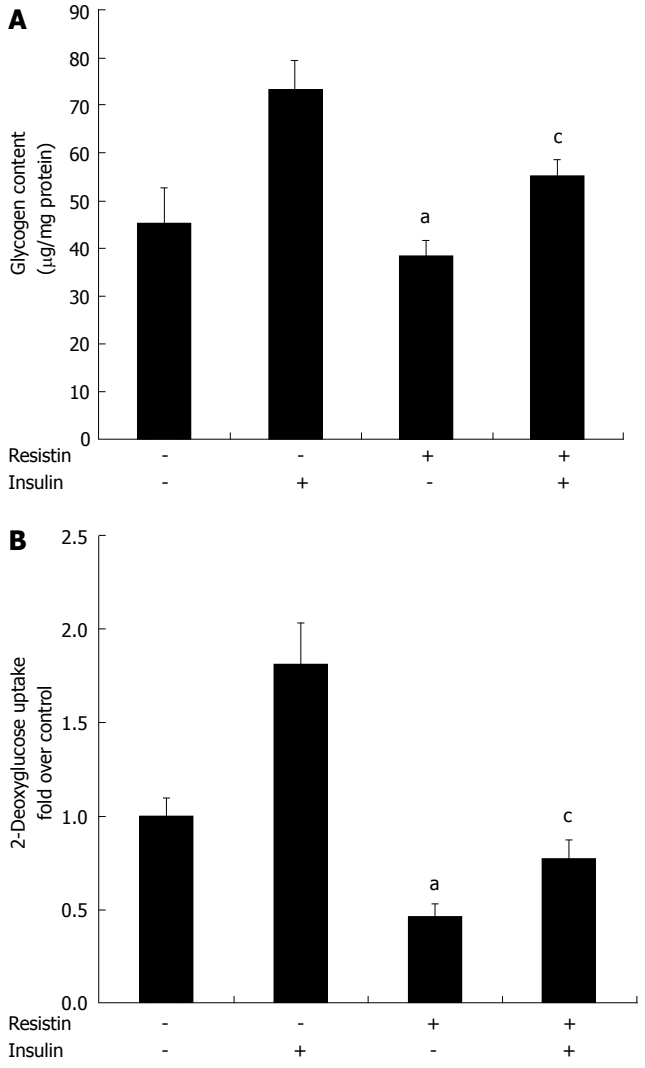


**Figure 3** Cellular insulin resistance induced by resistin after treatment of serum-starved H4IIE hepatocytes with 50 ng/mL resistin (**A**) and 100 nmol/L resistin (**B**). $^{a}P < 0.05$ *vs* negative control, $^{c}P < 0.05$ *vs* positive control.

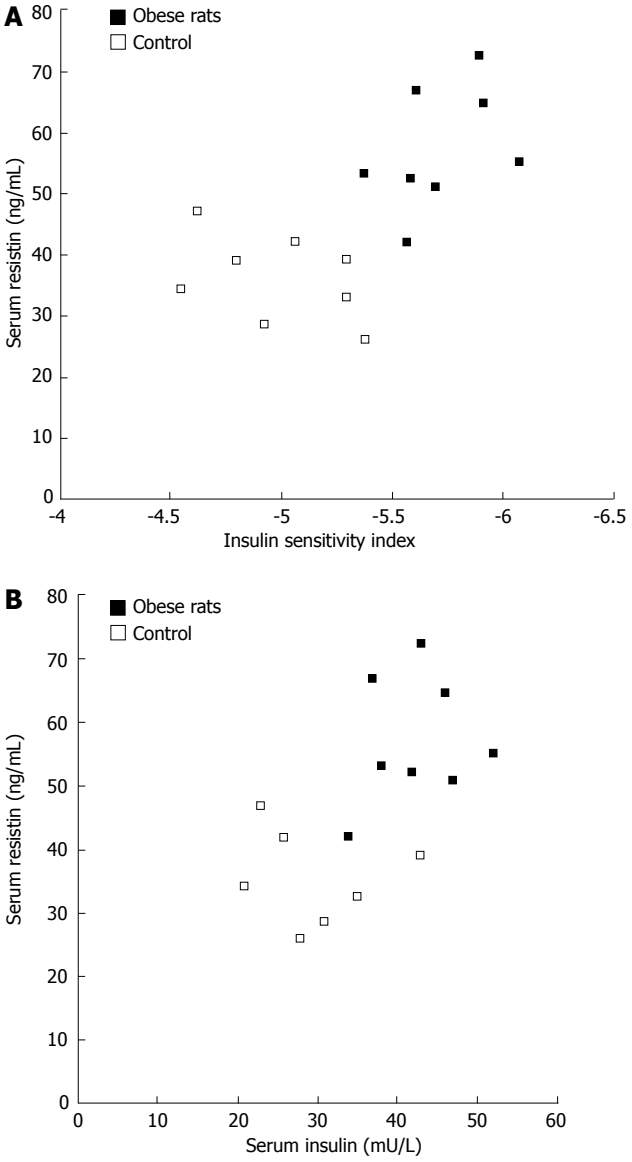


**Figure 4** Correlation of serum resistin with rat insulin sensitivity index (**A**) and insulin levels (**B**).

to provide a link between obesity and diabetes[3,4]. Under conditions of insulin resistance and type 2 diabetes, fat tissue is subjected to increased levels of insulin, which may have a major impact on adipokine levels[24,25]. Studies have verified that circulating levels of insulin are correlated with specific adipokines in rodents and humans, with interleukin-6 and leptin levels showing a consistently positive association with insulin levels[24-26]. However, the association of resistin with insulin remains contradictory[27].

Resistin is one of the adipocytokines secreted by adipose tissue and has been shown to modulate both glucose and lipid metabolism *in vivo* and *in vitro*[3,4]. In L6 rat skeletal muscle cells, it has been shown that resistin does not alter insulin receptor signaling but affects insulin-stimulated glucose uptake, presumably by decreasing the intrinsic activity of cell surface glucose transporters[7,8]. In mature 3T3-L1 adipocytes, resistin reduces insulin-stimulated glucose uptake by activating SOCS3, which is an inhibitor of insulin signaling[9]. In addition, it was reported that resistin takes part in insulin resistance in resistin fat-specific transgenic rats by releasing free fatty acids (FFA) from adipose tissue[28].

In the present study, resistin expression and secretion were increased during 3T3-L1 preadipocyte differentiation and resistin mRNA was undetectable in undifferentiated 3T3-L1 cells but was evident by d 4 after the induction of differentiation into adipocytes. The highest expression of resistin mRNA was detected on d 8 after induction of differentiation. Insulin had a marked suppressive effect on resistin mRNA levels in 3T3-L1 adipocytes and inhibited resistin secretion 6 and 8 d after induction of differentiation, suggesting that resistin does not play a role in insulin resistance.

Then, we investigated whether resistin impairs insulin sensitivity *in vitro*, showing that resistin could induce cellular insulin resistance in hepatocytes and myotubes. That is a paradox, because resistin is not regulated by insulin but induces insulin resistance[11]. If both are correct, they will form a deadly insulin-resistin-insulin sensitivity positive feedback loop.

To confirm which one plays the primary role *in vivo*, we analyzed the relationship between serum resistin and

insulin and their sensitivity in diet-induced obese rats. Diet-induced insulin resistance is a relevant model for the most common form of insulin resistance in humans[29]. In our study, serum resistin strongly correlated with rat insulin sensitivity and resistin was positively correlated with insulin, suggesting that insulin could not inhibit resistin secretion *in vivo*. A number of factors can regulate resistin secretion, such as glucose, epinephrine, and somatropin[27,30]. Therefore, insulin may regulate resistin although it is not the major regulator.

In summary, insulin inhibits resistin secretion while resistin induces insulin resistance. Serum resistin correlates with rat insulin sensitivity, meaning that insulin is not the major regulator of resistin. Resistin may play a role in diet-induced insulin resistance by inducing insulin resistance in hepatocytes and myotubes.

## COMMENTS

### Background

Type 2 diabetes mellitus is closely associated with obesity. Resistin is a recently identified adipokine involved in insulin sensitivity and glucose tolerance. So the investigation of insulin and resistin interaction seems to be important.

### Research frontiers

In this article, resistin biological function and interaction of resistin to insulin were studied. We also demonstrated the secretion levels of resistin *in vivo* and *in vitro*.

### Innovations and breakthroughs

Resistin induces insulin resistance in hepatocytes, but insulin inhibits resistin secretion *in vitro*. Since serum resistin correlates with rat insulin sensitivity, insulin is not the major regulator of resistin and resistin may play a role in diet-induced insulin resistance by inducing insulin resistance in hepatocytes and myotubes.

### Applications

Resistin is a newly identified hormone secreted by adipocytes. Resistin induces insulin resistance both *in vivo* and *in vitro*. This establishes a new field in the pathogenesis of type 2 diabetes.

### Peer review

This paper discusses the regulatory mechanisms of resistin and insulin. The study is well designed and the paper is well written. The topic is of scientific value.

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*RAPID COMMUNICATION*

# Lymphangiogenic and angiogenic microvessel density in human primary sporadic colorectal carcinoma

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## Abstract

**AIM:** To investigate the distribution pattern of lymphatic vessels and microvessels in sporadic colorectal carcinoma (SCRC) and their relationship to metastasis and prognosis.

**METHODS:** The lymphatic vessel density (LVD) and microvessel density (MVD) in tumor tissue obtained from 132 patients with primary SCRC, including 74 with metastases and 58 without metastases, were evaluated by immunohistochemistry using antibodies directed against D2-40 and von Willebrand factor (vWF).

**RESULTS:** (1) The lymphatic vessels and microvessels at central portions of SCRC often had a reticular architecture with numerous tiny and ill-defined lumina, while those at tumor borders had large and open lumina. The LVD and MVD were both obviously higher in colorectal cancer patients with metastases than in those without ($P < 0.001$). (2) For each one lymphatic vessel increased, there was a 1.45-fold increase in the risk of metastasis in SCRC. The specificity and sensitivity of LVD in predicting metastasis or non-metastasis in SCRC were 71.62% and 56.90%, respectively, and the corresponding LVD was 5. For each one microvessel increased, there was a 1.11-fold increase in the risk of metastasis in SCRC. The specificity and sensitivity of MVD were 66.22% and 51.72%, respectively. (3) Double labeling immunohistochemistry showed D2-40 immunoreactivity to be specific for lymphatic vessels. (4) Univariate analysis indicated that high LVD, high MVD, as well as co-accounting of high LVD and high MVD were associated with patient's poor disease-free survival ($P_{uni} < 0.05$); multivariate analysis indicated that co-accounting of LVD and MVD was an independent prognostic factor of colorectal cancer.

**CONCLUSION:** D2-40 is a new specific antibody for lymphatic endothelial cells. Lymphogenesis and angiogenesis are commonly seen in SCRC, especially at tumor borders. The detection of LVD and MVD at tumor borders may be useful in predicting metastasis and prognosis in patients with SCRC, and, in particular, co-accounting of LVD and MVD might be a useful prognostic factor in SCRC.



**Key words:** Lymphangiogenesis; Angiogenesis; Lymphatic vessel density; Microvessel density; Sporadic colorectal carcinoma; Metastasis

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## INTRODUCTION

Lymphangiogenesis (lymph vessel growth) and angiogenesis (blood vessel growth) are critical processes for tumor growth, invasion, and metastasis. Angiogenesis has established its role in the development and progression of a variety of malignancies, playing a crucial role in the dissemination of tumor cells[1,2]. However, lymphatic spread of cancer cells to lymph nodes is an important early event in the metastasis of carcinoma[3]. Previous studies have been limited by the lack of specific lymphatic endothelial makers that allow to discriminate between lymphatics and blood vessels. Recently, the monoclonal antibody D2-40, which is directed against the oncofetal membrane antigen M2A that has been identified in ovarian carcinoma cell lines and germ-cell tumors[4], was reported to be a specific marker for lymphatic endothelium in normal and neoplastic tissue[5,6].

In this study, using the antibody D2-40 and von Willebrand factor (vWF), we investigated the distribution patterns of microvessles and lymphatic vessels and calculated the lymphatic vessel density (LVD) and

microvessel density (MVD) in sporadic colorectal carcinoma (SCRC). Moreover, we analyzed their relationship with tumor metastasis and disease prognosis. By double labeling immunohistochemistry we were able to confirm D2-40 specificity.

## MATERIALS AND METHODS

### Patients and specimens

A total of 132 colorectal carcinoma samples were obtained from the archives of the Department of Pathology, Cancer Hospital, Fudan University, from July 2004 to May 2005. Samples were derived from patients who were solely surgically treated without prior chemo- or radiotherapy. There were 77 men (58.3%) and 55 women (41.7%) with a median age of 57 years (range, 22-82 years), 46 patients with colon and 86 patients with rectum tumors. 74 patients (56.1%) had histologically confirmed lymph node metastases, whereas the remaining 58 patients (43.9%) were found to have no clinical or histopathologic evidence of lymph node involvement. According to the current World Health Organization classification, tumors of 14 (10.6%) patients were well differentiated, that of 96 patients (72.7%) were moderately differentiated and that of 22 (16.7%) patients were poorly differentiated. Follow-up for this cohort was updated to September 2006. Complete data were available for 127 (96.2%) patients, with a median follow-up time of 22 mo (range, 2-26 mo). At the end of follow-up, 18 (14.17%) patients had died of disease and 9 (7.09%) had developed distant metastases. The study was approved by the local ethical committee in Cancer Hospital of Fudan University.

### Immunohistochemistry

Tissues were fixed in 10% buffered formalin, processed, and stained with hematoxylin and eosin (H&E). H&E-stained slides of all samples were reviewed to confirm the diagnosis. One paraffin block with the bulk of tumor tissue was used for immunohistochemical studies. All slides showed the nonneoplastic colorectal tissue-carcinoma junction. Sections, 3 mm-thick, of formalin-fixed paraffin embedded tissues were cut and mounted on coated slides. The sections were deparaffinized in xylene and rehydrated in a descending ethanol series. Heat induced epitope retrieval techniques were used for antigen retrieval as follows: citrate buffer (pH 6.0) and a water bath at 95℃-98℃ for 30 min for D2-40, Tris-EDTA buffer (pH 8.0) and a water bath at 95℃-98℃ for 30 min for vWF. Sections were incubated for 10 min in 3% hydrogen peroxide to quench endogenous tissue peroxidase. The sections were immunostained with a monoclonal antibody (Clone D2-40, m3619; Dako Corp., Carpinteria, CA, USA) at a 1:100 dilution directed against D2-40 and a rabbit polyclonal antibody (A0082; Dako) at a dilution of 1:200 directed against vWF. Tissue sections were incubated with the primary antibody for 12 h at 4℃. After washing with phosphate-buffered saline, a Super picture secondary antibody (Zymed Lab Inc, CA, USA) and 3-3' diaminobenzidine detection kit (Dako) were used. A lymphangioma tissue sample served as a positive control and replacement of the primary antibody by PBS as a negative control. Thirty samples were picked randomly for double labeling immunohistochemistry (Histostain™-DS double labeling immunohistochemistry kit, Zymed). The sections were first subjected to D2-40 staining using BCIP/NBT as chromogenic agent, followed by a vWF staining using AEC as chromogenic agent.

### Evaluation of immunostaining and vessels counting

Immunohistochemical reactions for D2-40 and vWF were interpreted independently by two authors (Y.G. and Z.GH.) using a two-headed microscope. After scanning the immunostained section at low magnification (× 100), five areas of carcinoma with the greatest number of distinctly highlighted intratumoral lymphatic/vascular foci (hot spots) were selected and vessels were counted in a representative high magnification (× 200) field in each of these five areas. Single immunoreactive endothelial cells, or endothelial cell clusters separate from other microvessels, were counted as individual microvessels. Endothelial staining in large vessels with tunica media, and non-specific staining of nonendothelial structures, were disregarded in microvessel counts. Mean visual microvessel density for D2-40 and vWF was calculated as the average of five counts[7]. In double labeling immunohistochemistry, lymphatic vessels were amethyst and blood vessels were bright red.

### Statistical analysis

Mean differences in microvessel counts were compared with the use of "*t*" tests. The probability of differences between the high-vessel group and the low-vessel group in disease-free survival (DFS) was determined as a function of time by the use of Kaplan-Meier test, with significance probing by applying the log-rank test. We used multivariate regression analysis based on the Cox proportional hazard model to test the independence of these parameters to predict overall survival. Logistic regression analysis and ROC curve were used to determine specificity and sensitivity of LVD and MVD in assessment of metastasis in SCRC. Generally, $P$ value $< 0.05$ was regarded as significant. For all statistical procedures, SPSS v12.0 and Stata v7.0 software were used.

## RESULTS

### Expression pattern of lymphatic vessels in SCRC and correlation of LVD with clinicopathologic parameters

A tissue sample of lymphangioma was chosen as a positive control for D2-40 staining. As shown in Figure 1A, endothelial cells in this sample were found to be positive. In SCRC samples, definite lymphatic vessels were stained by D2-40. Lymphatic vessels at central portions were rare, even absent in some case, and often had a reticular architecture with numerous tiny and ill-defined lumina (Figure 1B). The LVD was higher at tumor borders (10.32 ± 4.94) than at central portions and had large and open lumina (Figure 1C). The LVD was obviously higher in the CRC samples with metastases (12.08 ± 4.96) than in those without (8.26 ± 4.08) ($P < 0.001$). There was no significant correlation between

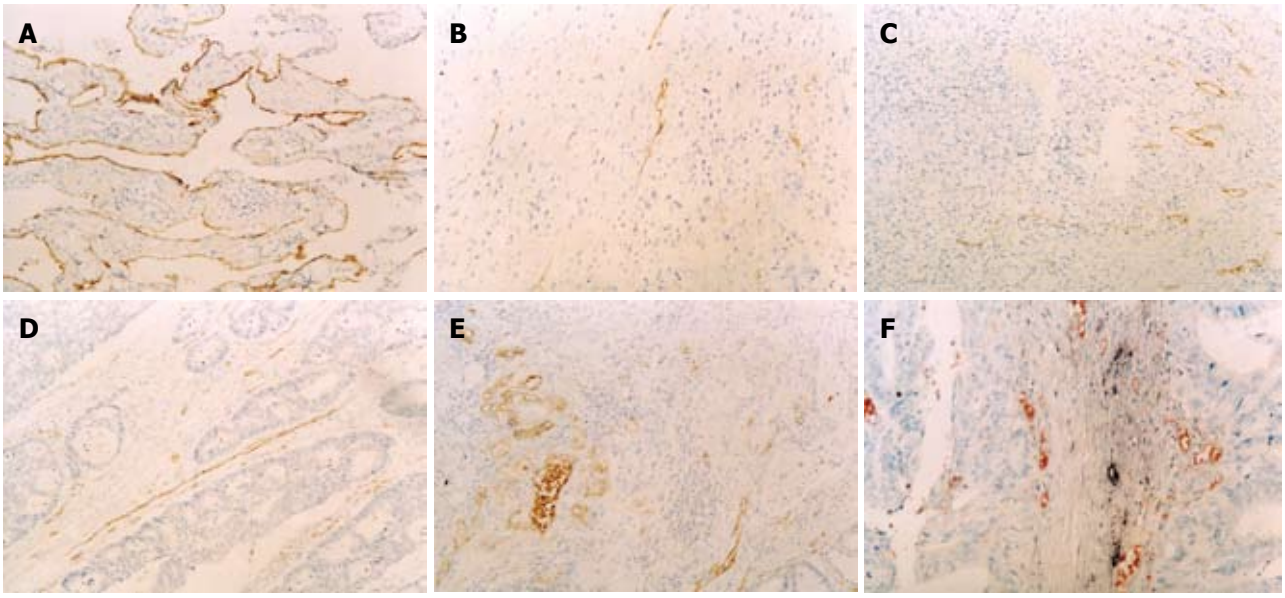


**Figure 1** Immunohistochemical stainings of D2-40 (**A**, **B**, **C** × 100), vWF (**D**, **E** × 100) and double labeling immunohistochemistry (**F** × 200, red: Blood vessels labled by vWF; amethyst: Lymphatic vessels by D2-40). **A**: Lymphangioma (positive control); **B-F**: Colorectal carcinoma.

**Table 1 Correlation of LVD/MVD with clinicopathologic parameters of SCRC**

| Clinico-pathologic features | *n* | LVD | | MVD | |
|---|---|---|---|---|---|
| | | mean ± SD | *P* | mean ± SD | *P* |
| Age (yr) | | | | | |
| <50 | 28 | 11.03 ± 5.71 | 0.396 | 19.58 ± 9.56 | 0.332 |
| ⩾ 50 | 104 | 10.14 ± 4.73 | | 21.92 ± 11.76 | |
| Gender | | | | | |
| Male | 77 | 10.50 ± 4.73 | 0.641 | 21.00 ± 11.74 | 0.609 |
| Female | 55 | 10.09 ± 5.27 | | 22.02 ± 10.82 | |
| Tumor size | | | | | |
| < 5 cm | 77 | 10.37 ± 4.84 | 0.904 | 22.26 ± 11.92 | 0.313 |
| ⩾ 5 cm | 55 | 10.26 ± 5.13 | | 20.24 ± 10.46 | |
| Location | | | | | |
| Right colon | 23 | 9.36 ± 3.95 | 0.323 | 23.36 ± 10.33 | 0.248 |
| Left colon | 23 | 9.52 ± 4.65 | | 18.06 ± 11.25 | |
| Rectum | 86 | 10.80 ± 5.23 | | 21.81 ± 11.55 | |
| Invasive depth | | | | | |
| Intra-deep muscular | 36 | 9.31 ± 5.82 | 0.15 | 21.74 ± 11.02 | 0.845 |
| Whole layer | 96 | 10.71 ± 5.82 | | 21.30 ± 5.76 | |
| Degree of differentiation | | | | | |
| Well | 14 | 8.38 ± 4.60 | 0.119 | 21.28 ± 9.38 | 0.962 |
| Moderately & poorly | 118 | 10.56 ± 4.95 | | 21.44 ± 11.58 | |
| Lymph node metastasis | | | | | |
| Yes | 74 | 12.01 ± 4.90 | < 0.001 | 24.00 ± 11.98 | 0.003 |
| No | 58 | 8.18 ± 4.12 | | 18.14 ± 9.58 | |

LVD: Lymphatic vessel density; MVD: Microvessel density.

LVD with age, gender, tumor size, location, degree of differentiation, or invasive depth ($P > 0.05$) (Table 1).

### Logistic regression analysis and ROC curve of LVD in SCRC

A logistic model was built with LVD as an independent and metastasis as dependent variable (Prob > chi2 = 0.0000, Pseudo R2-0.1176). According to the analysis for of the logistic model, we found an OR value of 1.45, meaning that for each lymphatic vessel there is an 1.45-fold increase in the risk of metastasis in SCRC. The specificity and sensitivity of LVD in predicting metastasis or non-metastasis in SCRC were 71.62% and 56.90%, respectively, and the corresponding LVD was 5 (Figure 2A).

### Expression pattern of blood microvessels in SCRC and correlation of MVD with clinicopathologic parameters

There were abundant microvessels labeled by vWF in SCRC, with the similar distribution pattern to lymphatic vessels. The distribution feature and quantity of microvessels were different within one tumor sample. That is, microvessels at surrounding part of tumor were abundant (mean = 21.24 ± 11.98) and had large and open lumina (Figure 1D). But, microvessels at central portions were seldom and often had a reticular architecture with numerous tiny and ill-defined lumina (Figure 1E). The MVD was obviously higher in the colorectal carcinoma samples with metastasis (23.74 ± 12.02) than in those without (18.00 ± 9.44) ($P = 0.003$). There was no significant correlation between MVD with age, gender, tumor size, location, degree of differentiation, or invasive depth ($P > 0.05$) (Table 1).

The specificity of D2-40 for lymphatic vessels and vWF for microvessels was confirmed by double labeling immunohistochemistry. There was no cross-reaction between the two antibodies. Lymphatic vessels and microvessels distributed separately in tumor borders without obvious relationship (Figure 1F).

### Logistic regression analysis and ROC curve of MVD in SCRC

A l-Logistic model with MVD as an independent and metastasis as the dependent variable could be established (Prob > chi2 = 0.0022, Pseudo R2 = 0.0519). According to this model, we found an OR of 1.11, meaning that for each microvessel there is an 1.11-fold increase in the risk of metastasis in SCRC. According to ROC curve analysis, the specificity and sensitivity of MVD in predicting metastasis or non-metastasis in SCRC were 71.62% and 56.90%, respectively (Figure 2B).

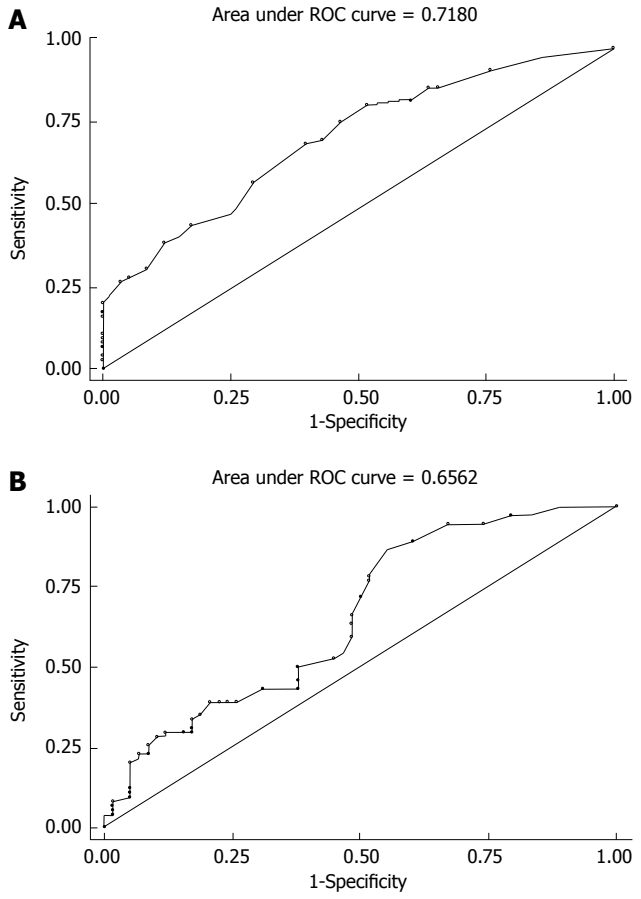


**Figure 2** ROC curve of LVD (**A**) and MVD (**B**).

### *Univariate survival analysis*

LVD = 5 was defined as demarcation value according to the ROC curve of LVD. LVD > 5 was high LVD group and LVD < 5 was low LVD group. The median of MVD (median = 9.33) was defined as demarcation value. MVD > 9.33 was high MVD group and MVD < 9.33 was low MVD group. LVD > 5 and MVD > 9.33 was co-high group. In univariate analysis with Kaplan-Meier for disease-free survival (DFS), high LVD ($P$ = 0.0303), high MVD ($P$ = 0.0196), co-high LVD and MVD ($P$ < 0.0001) were associated with patient's poor DFS ($Puni$ < 0.05) (Figure 3).

### *Multivariate survival analysis*

All factors were brought into a Cox regression model, including some clinico-pathologic parameters such as patients' age, gender, tumor size, location, degree of differentiation, invasive depth and metastasis, LVD, MVD, and co-accounting of LVD and MVD. Multivariate analysis indicated that besides metastasis ($P$ = 0.004), gender ($P$ = 0.012), and location ($P$ = 0.028), co-accounting of LVD and MVD was an independent prognostic factor of colorectal carcinoma ($P$ = 0.014).

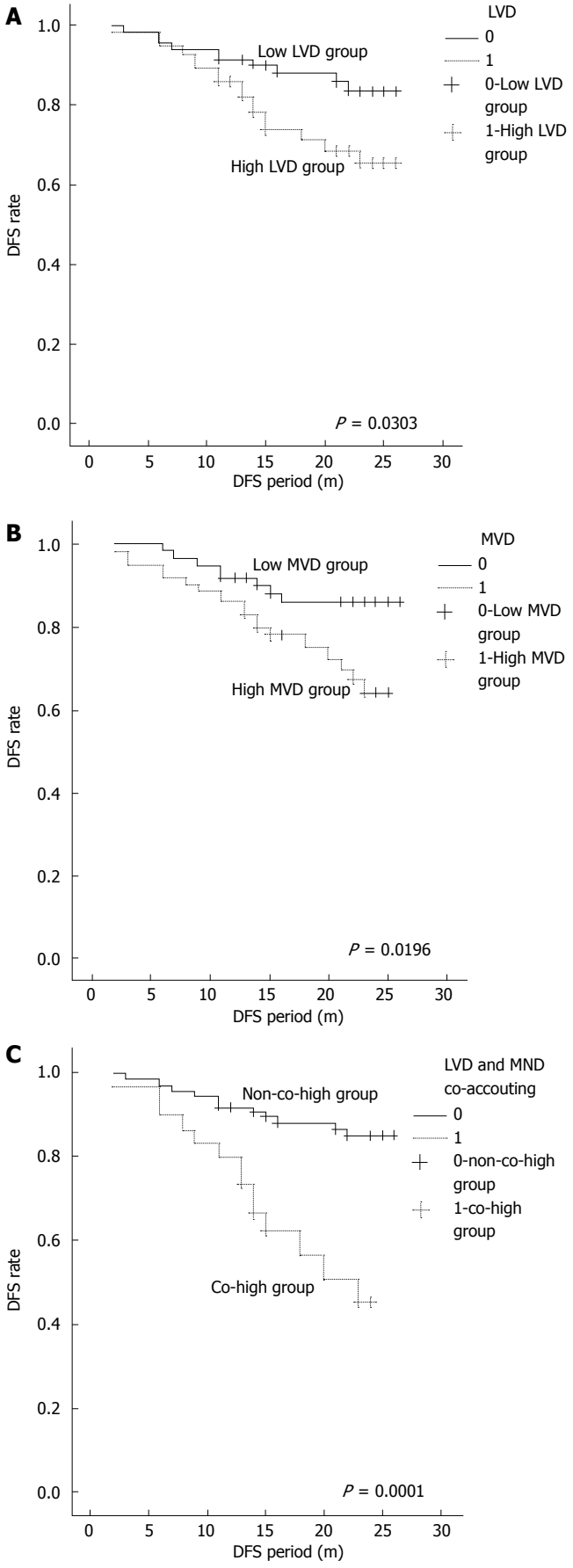


**Figure 3** Survival curve of LVD (**A**), MVD (**B**) and co-accounting of LVD and MVD (**C**).

## DISCUSSION

This study is one of the first attempts to quantify colorectal carcinoma lymphangiogenesis and angiogenesis in the same sample by microvessel density using the novel lymphatic marker D2-40 and vWF. We compared the distribution pattern and density of lymphatic vessels and

blood vessels, and related the results to clinicopathologic parameters and the outcome of colorectal carcinoma. Moreover, specificity of the two antibodies was confirmed by double labeling immunohistochemistry.

### *Significance of lymphangiogenesis in SCRC*

The lymphatic system is the primary pathway of metastasis for most human cancers. Lymphangiogenesis refers to the development and proliferation of new lymphatics from host vessels. Recently, antibodies specific for lymphatic endothelium have become available, providing important new insights into the process of tumor-associated lymphangiogenesis and its possible clinical relevance. Many studies have reported that tumors are able not only to induce lymphangiogenesis, but also to enhance lymphatic metastasis[8,9].

There are some antibodies for lymphatic endothelium now, including VEGFR-3/flt4 (vascular endothelial growth factor receptor 3/fms-like tyrosine kinase 4)[10], LYVE-1 (lymphatic endothelial hyaluronan receptor)[11], Prox-1[12], CD31[13], and podoplanin[14]. VEGFR-3, the receptor for vascular endothelial growth factors (VEGFs) C and D, is expressed on lymphatic endothelium and may play a role in lymphangiogenesis[15,16]. But, some studies indicated that VEGFR-3 was also involved in blood vessel angiogenesis in the adult and it was not a specific antibody for lymphatic endothelium[17]. CD31 also stained both in blood vessel and in lymphatic vessel. But, the monoclonal antibody D2-40 is a highly selective marker of lymphatic endothelium in sections of both frozen and formalin-fixed paraffin-embedded normal and neoplastic tissues. In a direct comparison of D2-40 and CD31 on paraffin sections of a series of tumors derived from lymphatic endothelium (lymphangiomas) and blood vessel endothelium (hemangiomas), D2-40 stained all lymphagiomas (10/10) but no hemangiomas (0/10). Conversely, CD31 stained a fraction of lymphangiomas (5/10) but all hemangiomas (10/10)[13].

Pusztaszeri *et al*[18] found D2-40 to be a sensitive and relatively specific marker for lymphatic endothelium in all parenchymatous organs. It stained lymphatic endothelium only and it has been demonstrated to recognize tumor-associated lymphatic vessels in many tumors[19,20]. Lymphatic vessels stained by D2-40 in our study often had a defined lumen with thin walls lacking erythrocytes, which were all the features of lymphatic vessels. There was no cross-reaction between D2-40 for lymphatic vessels and vWF for microvessels in double labeling immunohistochemistry. D2-40 is specific for lymphatic vessels[21,22].

We also found lymphatic vessels to show a different distribution between tumor central portions and tumor borders in SCRC. Lymphatic vessels at central portions were seldom, even absent in some cases, but abundant at tumor borders. This phenomenon may be due to the inside pressure of the tumor. The LVD was obviously higher in the cases of colorectal carcinoma with metastasis than that in cases without, indicating that tumor cells might migrate from a primary site to lymph nodes through lymphatic vessels at the tumor borders. The increase of LVD was related to lymph nodes metastasis in SCRC. According to the logistic model and ROC curve analysis, we found an OR of 1.45, meaning that for each lymphatic vessel there is a 1.45-fold increase in the risk of metastasis in SCRC. The specificity and sensitivity of LVD in predicting metastasis or non-metastasis in SCRC were 71.62% and 56.90%, respectively, and the corresponding LVD was 5. The univariate analysis showed high LVD to be associated with patient's poor DFS. Thus, LVD was an important factor to predict metastasis and prognosis for the patients with SCRC. Lymphatic vessels were composed of a single, non-fenestrated endothelial layer with wide and irregular lumina. Endothelial cells, with scant cytoplasm were often overlapping. But there were infrequent interendothelial tight junctions and no basement membrane and encircling pericytes on them. The morphology of lymphatic vessels differed from that of blood microvessels consequently made it was easy for fluid, particles, and tumor cells to pass into the lymphatic vessels. The surface area between tumor cells and lymphatic endothelial cells increased with the enhancement of LVD, which promoted the migration of tumor cells to lymph nodes[2]. So lymphangiogenesis is associated with an increased incidence of lymph nodes metastasis, and it is possible that this step is essential to the metastatic process. Recently, some studies on gastric cancer[23] and breast cancer[24,25] demonstrated LVD to be correlated with lymph nodes metastasis.

### *Significance of angiogenesis in SCRC*

Angiogenesis, the formation of new blood vessels from the endothelium of the existing vasculature, is fundamental in tumor growth, progression and metastasis, especially for colorectal carcinoma[26]. The complex network of tumor blood microvessels guarantees adequate supply of tumor cells with nutrients and oxygen and provides efficient drainage of metabolites. In addition to primary tumor growth, metastatic tumor growth depends upon neovascularization in at least two steps: First, malignant cells must exit from a primary tumor into the blood circulation after the tumor becomes neovascularized. Second, after arrival at distant organs, metastatic cells must again induce angiogenesis for a tumor to expand to a detectable size[27,28]. Zhao *et al*[29] suggests MVD as one of the important prognostic factors for gastric cancer patients by immunohistochemical staining of endothelial protein factor Ⅷ-related antigen. Romani *et al*[30] evaluated retrospectively the effect of CD105-assessed (a marker of neovascularization in solid malignancies) angiogenesis on the risk of developing metastatic disease in colorectal cancer. One hundred and twenty-five paraffin-embedded samples were analyzed by immunohistochemical methods using CD105 monoclonal antibody. The CD105-vessel count was found to be strongly correlated with the occurrence of metastatic disease. The median CD105-positive vessels in patients with and without metastatic disease were 24.7 and 13.2 vessels/mm$^2$, respectively ($P < 0.001$). For each one microvessel increase in the vessels count per 400 × field, there was a 1.42-fold increase in the risk of metastatic disease ($P < 0.001$). We found the distribution feature and quantity of microvessels were different within one tumor sample labeled by vWF, which is similar to the distribution pattern of lymphatic vessels.

That is, microvessels at surrounding part of tumor were abundant (mean = 21.24 ± 11.98) and had large and open lumina. In contrast, microvessels at central portions were less frequent and often had a reticular architecture with tiny and ill-defined lumina. The MVD was obviously higher in samples from colorectal carcinoma patients with metastasis than in those without. Our results indicated metastasis of SCRC to be associated with MVD. According to the logistic model, we found an OR of 1.11, meaning for each microvessel there is an estimated 1.11-fold higher risk of metastasis in SCRC. According to ROC curve analysis, the specificity and sensitivity of MVD in predicting metastasis or non-metastasis in SCRC were 71.62% and 56.90%, respectively. The univariate analysis showed high MVD to be associated with patient's poor DFS. The lymphatic system was the most common pathway of metastasis for SCRC, accounting 60%. And the second primary pathway was blood vessels. Compared with LVD, the specificity and sensitivity of MVD in predicting metastasis or non-metastasis was lower. But MVD still played a role in roughly predicting prognosis of patients with SCRC.

Lymphangiogenesis and angiogenesis are essential for metastasis of tumor cells. We evaluated the specificity (71.62% *vs* 66.22%) and sensitivity (56.90% *vs* 51.72%) of LVD and MVD in SCRC. The specificity and sensitivity of LVD was slightly higher than that of MVD. Thus, neither LVD nor MVD alone were sufficiently specific and sensitive to predict metastasis or prognosis. We thus combined LVD with MVD to co-account. Univariate analysis indicated that co-accounting of high LVD and high MVD were closely associated with patient's poor DFS and multivariate analysis indicated besides metastasis, gender and location, co-accounting of LVD and MVD to be independently predictive. Thus, evaluating lymphangiogenesis and angiogenesis are thought to be clinically important, particularly for the estimation of the metastatic risk and prognosis.

In summary, D2-40 is a new specific antibody for lymphatic endothelial cells. Lymphogenesis and angiogenesis are commonly seen in SCRC, especially at tumor borders. The detection of LVD and MVD at tumor borders may be useful in predicting metastasis and prognosis in patients with SCRC, and especially, the co-accounting of LVD and MVD might be used as a prognostic factor of SCRC.

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## COMMENTS

### Background

More and more researchers focus on the importance of lymphogenesis in tumorigenesis and metastasis because of the development and update of new markers for lymphatic vessels. It is a common phenomenon that metastatic local lymph node in sporadic colorectal carcinoma (SCRC), but the relationship between the lymphogenesis and metastasis is not clear.

### Research frontiers

D2-40 is a new specific marker for lymphatic endothelium in normal and neoplastic tissue. Recent studies demonstrated D2-40 to be probably useful for diagnosis of malignant mesothelioma and research on lymphatic spread of cancer cells.

### Innovations and breakthroughs

We investigated the role of lymphogenesis and angiogenesis using D2-40 and their distribution patterns in Sporadic colorectal carcinoma (SCRC). Moreover, we assessed how to predict metastasis by the increase of Microvessel density (MVD) and Lymphatic vessel density (LVD) and determined specificity and sensitivity.

### Applications

Our study found LVD and MVD to be related to prognosis of SCRC. We calculated the risk ratio in predicting metastasis in SCRC by statistical procedures. In the future, we will be able to give an estimate on the prognosis and the survival of patients with colorectal carcinoma by accounting LVD and MVD.

### Terminology

SCRC: Sporadic colorectal carcinoma; LVD: Lymphatic vessel density; MVD: Microvessel density.

### Peer review

The results of this paper are reliably to the conclusions. The innovative and significant points conform to the background, objectives, materials and methods, results and conclusions. There is no conflict of interest, nor ethics problems. This is significant research.

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*RAPID COMMUNICATION*

# Expression of phosphatase and tensin homolog deleted on chromosome ten in liver of athymic mice with hepatocellular carcinoma and the effect of Fuzheng Jiedu Decoction

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## Abstract

**AIM:** To explore the expression of phosphatase and tensin homolog deleted on chromosome ten (PTEN) in liver of athymic mice with hepatocellular carcinoma (HCC) and the effect of Fuzheng Jiedu Decoction (FJD).

**METHODS:** Forty eight male BALB/c athymic mice models were built by Bel-7402 with an indirect method. After 24 h of postoperation, the 48 athymic mice were distributed randomly into 4 groups: A, B, C, D, each group had 12 athymic mice. Group A were treated by intragastric administration with FT207 (Tegafur) for 4 wk. Group B, C and D were treated by intragastric administration with FJD (complex prescription of Chinese crude drug) that had been delegated into 3 kinds of density as the low, middle, and high for 4 wk. At last, athymic mice were put to death, live time, volume of tumors, exponent of tumors and the tumor metastasis in livers were observed; and PTEN was detected in hepatic tissue, latero-cancer tissue and cancer tissue by immunohistochemistry.

**RESULTS:** Four weeks later, the total survival rate in treatment group (A + B + C) was 50% and higher than the control group (0%) treated by FT207, ($P < 0.01$). The survival rate in group A, B, C was higher than in group D, and except group A with D, there was significant differentces (Fisher's Exact Test $P = 0.05$ or 0.01). And no differences were observed between the treatment groups and the control group in volume of tumors and exponent of tumors ($P > 0.05$). Tumor metastasis in livers of the treatment group was less than the controls (Fisher's Exact Test, $P = 0.021$). The result of immunohistochemistry showed that the intensity of PTEN in latero-cancer tissue was the highest, and then the hepatic tissue, the lowest was cancer tissue (Kruskal-Wallis test, $\chi^2 = 60.67$, $P = 0.000$). It also showed that the intensity of PTEN in treatment groups (A, B, C) was higher than the control group (D) ($F = 5.90$, $P = 0.002$ in hepatic tissue and $F = 15.99$, $P = 0.000$ in latero-cancer tissue and $\chi^2 = 26.08$, $P = 0.000$ in cancer tissue), and group B is the highest in the treatment groups ($P < 0.05$, $r = 0.01$. respectively). However, there was no significant statistic difference between group A and group C ($P > 0.05$).

**CONCLUSION:** FJD can prolong the survival time and decrease tumor metastasis in livers of these experimental mice. Mechanisms of FJD healing HCC may partially be explained by enhancing the expression of PTEN in liver.



**Key words:** Phosphatase and tensin homolog deleted on chromosome ten; Athymic mice; Hepatocellular carcinoma; Fuzheng Jiedu Decoction

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## INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the major cancer killers. Although surgical resection, liver transplantation and percutaneous ablation are considered as effective treatment for HCC[1-3], Traditional Chinese Medicine (TCM) has been widely used as combined therapies in treating the disease in China. According to the theory of TCM, the main mechanism of HCC

is deficiency of vital qi and exuberant pathogens, thus strengthening body resistance and disintoxication is the major method of treating HCC[4]. The previous study demonstrated that reduced expression levels of PTEN are involved in the pathogenesis of HCC. Moreover, decreased phosphatase and tensin homolog deleted on chromosome ten (PTEN) expression was correlated with tumor progression and poor prognosis in patients with HCC[5-7], whether TCM can down-regulate the expression of PTEN in HCC is still unknown. The aim of our study is to explore the effect of Fuzheng Jiedu Decoction (FJD), complex prescription of Chinese crude drug in treating BALB/c athymic mice with HCC, as well as the expression of PTEN. We proclaim that the animal study was acknowledged by the Ethical Committee of The First Affiliated Hospital of Sun Yat-Sen University committee in the materials and methods section. The results are reported as following.

## MATERIALS AND METHODS

### Study animals and environmental conditions

Forty eight BALB/c athymic male mice, 4-6 wk, were purchased from The Experimental Animal Center of Traditional Chinese Medicine university of Guangzhou. The animals were housed individually in cages and kept in a room maintained at a temperature of 23 with a relative humidity (RT) of 55 with a 12-h/12-h light/dark cycle. Solid rodent chow and tap water were given ad libitum.

### Drugs and reagents

FJD (Application: 200710026976.6) consists of the following indegredients: Ezhu (Rhizoma Curcumae) 15 g, Banzhilian (Herba Scutellariae Darbatae ) 30 g, *etc*. They were decocted routinely and then made into a final concentration of 2 g/mL which extracted and prepared by college of pharmacy of Traditional Chinese Medicine University of Guangzhou. The prepared herbal pieces were purchased from Guangzhou City Pharmacy Company and consistent with the requirement of Pharmacopoeia of the People's Republic of China[8]; FT207 parenteral solution (Tegafur parenteral solution), 5 mL/0.2 g per ramus, Shandongqilu production (production batch number 06020032); rabbit anti-human PTEN polyclonal antibody (production batch number 60182150), Power Vision™ Two-Step Histostaining Reagent (production batch number 125135), were bought from Beijing Zhongshan Jinqiao biotechnology limited company.

### Preparation of animal model

The athymic mice with indirect orthotopic transplantation tumor models were established in accordance with the method by Dr. Zheng Jianhua[9]. The animals were inoculated Bel-7402 hepatoma carcinoma cell with concentration of 1 million cells/mL on their waist and back, until subcutaneous transplantation tumor grew to diameter 1 cm, then cut down the tumor. Remove necrotic tissue in the tumor and cut into pieces about 1 $mm^3$ in Hanks liquid. Anesthetize the athymic mice in abdominal cavity with Pentobarbital 45 mg/kg weight, transverse incise the left upper quadrant, expose liver, take 1 piece of tumor tissue, use bodkin pinhead (20° angle of slope, deep 3 mm) imbed the tumor tissue in deep part of hepatic lobes parenchyma of athymic mouse in *ex vivo* 40 min, compresse the incision to stop bleeding then close abdomen.

### Grouping and the treatment of the animals

Athymic mice were raised in SPF condition in divided cages. After 24 h of postoperation, the 48 athymic mice were distributed randomly into 4 groups, every group has 12 mice. Low concentration group (A): drench the Ganaifang (0.25 g Chinese crude drug/mL, 0.2 mL/10 g weight) with the dose of 10 times human unit kilogram weight. Middle concentration group (B): drench the Ganaifang (0.5 g Chinese crude drug/mL, 0.2 mL/10 g weight) with the dose of 20 times human unit kilogram weight. High concentration group (C): drench the Ganaifang (1.0 g Chinese crude drug/mL, 0.2 mL/10 g weight) with the dose of 40 times human unit kilogram body weight. Chemotherapy group (D): drench the FT207 parenteral solution (Tegafur parenteral solution) (8 mg/mL, 0.2 mL/10 g weight) with the dose of 5 times human per day.

Record live time of each athymic mouse in the process of observation. When the treatment was ended, get blood from athymic mice eyeball, and put them to death, then dissect them, meanwhile record the tumor volume, tumor index number (the weight of the tumor/the weight of the mouse), fix the normal hepatic tissue, latero-cancer tissue and cancer tissue in 4% neutral formalin, sent to pathology laboratory to make paraffin imbedding microtome sections (4 mm thick serial sections) fixed in silicification glass.

### Pathologic detection

Histologic detection: dying with the routine hematoxilin -eosine (HE).

Immunohistochemistry detection: 4 μm thick paraffin sections were baked at 65℃ until get deparaffinage and hydration; incubate in 3% Hydrogen Dioxide about 5-10 min to inactivate the endogenous peroxidase; microwave repairs the antigen; sealed by 10% normal goat serum; PTFN multiclone rabbit antibody (1:100) was incubated overnight at 4℃, washed by PBS 2 min for 3 times; dropwise goat anti-rabbit IgG antibody-HRP multimer, incubated about 30 min at 37℃, washed by PBS, 2 min × 3 times; DAB coloration; washed thoroughly by distilled water. In the same time, PBS was used as first-antibody and second-antibody in negative control.

Evalution of coloration result: using Bresalier[10] semiquantitative formula to judge coloration result. Selecting randomly 10 fields of vision from every section when enlarged 200 times, then we classified the cell coloration intensity into four categories: Negative, cell hasn't colored (0); Cell has colored buff (1); claybank (2); chocolatebrown (3). Count the number of field of vision of each intensity, and according to the formula calculate the average coloration intensity. IS (intensity score) = $\sum[(0 \times F0) + (1 \times F1) + (2 \times F2) + (3 \times F3)]$, $F = n/10$ ($n$ = 0, 1, 2, 3 the number of field of vision of different score). Two people read the section two times with double

**Table 1 Survival time in different groups**

| Group | *n* | Survival days mean ± SD | Survival rate (%) 3 wk | 4 wk |
|---|---|---|---|---|
| A | 12 | 23.67 ± 4.92 | 10/12 (83.3) | 5/12 (41.7) |
| B | 12 | 26.08 ± 2.31 | 12/12 (100)[a] | 6/12 (50)[a] |
| C | 12 | 25.92 ± 2.71 | 12/12 (100)[a] | 7/12 (58.3)[b] |
| D | 12 | 18.83 ± 2.29 | 7/12 (58.3) | 0/12 (0) |

[a]$P < 0.05$, [b]$P < 0.01$ *vs* group D.

**Table 2 Volume and index of tumors (mean ± SD)**

| Group | *n* | Volume ($mm^3$) | Index |
|---|---|---|---|
| A | 12 | 466.43 ± 645.66 | 0.0037 ± 0.0403 |
| B | 12 | 215.91 ± 275.23 | 0.0305 ± 0.0403 |
| C | 12 | 325.23 ± 464.30 | 0.0313 ± 0.0436 |
| D | 12 | 309.7 ± 309.72 | 0.0462 ± 0.0296 |

**Table 3 Tumor metastasis in livers**

| Group | Rate of tumor metastasis in liver (%) |
|---|---|
| A + B + C | 23/36 (63.9)[a] |
| A | 7/12 (58.3)[c] |
| B | 9/12 (75) |
| C | 7/12(58.3)[c] |
| D | 12/12(100) |

[a]$P < 0.05$ *vs* group D; [c]$P < 0.05$ *vs* group D.

blind method, calculate IS for each time, and get the two times average as the result.

### Statistical analysis

Using SPSS 14.0 statistic software, variance analysis and rank-sum test were used for measurement data; chi square test and rank-sum test were used for numeration data.

## RESULTS

### General situation

The dissection witness that achievement ratio of making model is 100%; at the end of the experiment, get 48 utility pathologic samples. Most tumors were enormous, some were accompanied multilesions in the liver and/or lung.

It was demonstrated that the model was stable and facility, consistent with dissection characteristic of human liver cancer.

### Survival state

After 3 wk of drug intervention, the total survival rate in treatment group (A + B + C) was 94.4% and much higher than the chemotherapy group (58.3%) ($P = 0.007$, Fisher's Exact Test). Though the survival rate in group A was higher than that of group D, no differences were observed between them ($P > 0.05$). The survival rate in group Band C is higher than in group D, there was a significant difference ($P = 0.014$). 4 wk later, the total survival rate in treatment group (A + B + C) was 50% and much higher than the chemotherapy group (0%) ($P = 0.02$). Though the survival rate in group A was higher than that of group D, no difference were observed between them ($P > 0.05$). The survival rate in group B and C was higher than in group D, there was significant difference, ($P < 0.05$ or 0.01) (Table 1).

**Table 4 Expression intensity of PTEN in the 3 kinds of tissue ($\alpha = 0.01$)**

| Kind of tissue | *n* | PTEN intensity median |
|---|---|---|
| Normal hepatic tissue | 48 | 1.00 |
| Latero-cancer tissue | 48 | 1.31 |
| Cancer tissue | 48 | 0.23 |

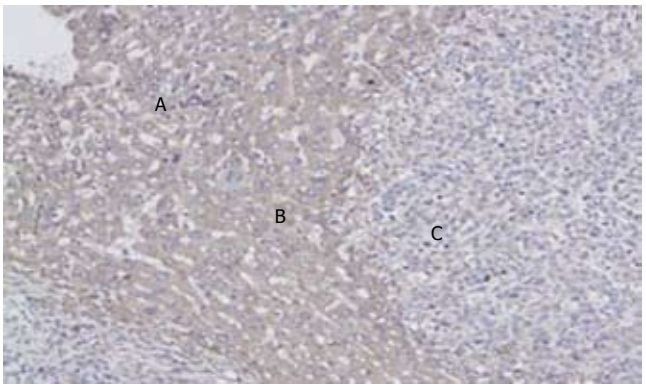


**Figure 1** Intensity of PTEN: Latero-cancer tissue. A: > cancer tissue; B: > hepatic tissue; C: Two-Step, × 100.

### Tumor state

No differences was observed between the group A, group B, group C and the group D in volume and index of tumors ($P > 0.05$) (Table 2).

### The state of tumor metastasis in liver

After the transplantation tumors in liver of each mouse was taken out, then the remain liver were observe by microscope, if it existed hepatoma carcinoma cell, We diagnosed the liver had the metastases. Tumor metastasis in livers of group A and group C were both 58.3% (7/12) and much less than the group D 100% (12/12) $P = 0.037$. Tumor metastasis in livers of group A, group B and group C was 63.9% (23/36) and much less than the group D ($P = 0.021$), but there was no difference among group A, group B and group C (Table 3).

### The expression intensity of PTEN in different tissues

Three groups were compared with each other applied Kruskal-Wallis test, $\chi^2 = 60.67$, $P = 0.000$. The result showed that the intensity of PTEN in latero-cancer tissue was the highest, and then the normal hepatic tissue, the lowest was cancer tissue (Table 4, Figures 1 and 2).

### Interclass analyses positive expression intensity of PTEN in three kinds tissue

Tested by One-way ANOVA, LSD, Tamhane Test was used in normal hepatic tissue and latero-cancer tissue, and Mann-Whitney *U* in cancer tissue. in normal hepatic tissue

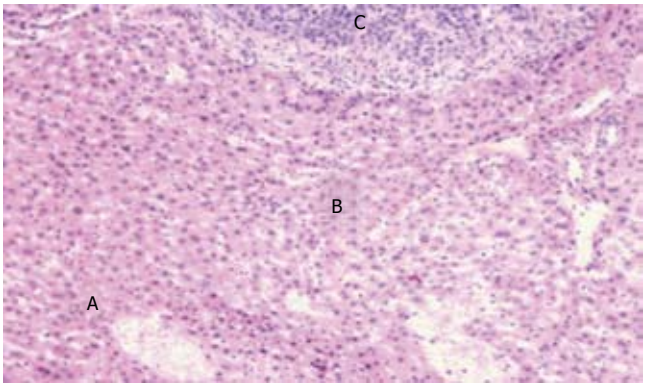


**Figure 2** A: Hepatic tissue; B: Latero-cancer tissue; C: Cancer tissue, (HE, × 100).

(Test of Homogeneity of Variances, Levene Statistic = 2.18, $P = 0.104$; and One-way ANOVA Test, $F = 5.90$, $P = 0.002$), A > D, $P = 0.001$; B > D, $P = 0.031$; C > D, $P = 0.001$. It represented that the intensity of PTEN in Chinese crude drug group was higher than the Tegafur group. However, there was no significant statistic difference among Chinese crude drug group in difference concentrations ($P > 0.05$). In latero-cancer tissue, (Test of Homogeneity of Variances, Levene Statistic = 3.09, $P = 0.037$; and One-way ANOVA Test, $F = 15.99$, $P = 0.000$), A < B, $P = 0.170$; A > C, $P = 0.091$; B > C, $P = 0.017$; C > D, $P = 0.028$, it showed that expression intensity of PTEN under the intervention of Chinese crude drug was higher than that of Tegafur, the expression intensity of PTEN in medium dose group was the highest ($P = 0.05$). In cancer tissue, A < B, U = 30.5, $P = 0.016$; A > C, U = 44, $P = 0.104$; A > D, U = 24, $P = 0.005$; C > D, U = 18, $P = 0.002$; B < C, U = 9.5, $P = 0.000$; B > D, U = 4, $P = 0.000$. It represents that the intensity of PTEN in medium dose group was highest among the 3 kinds of tissue. In cancer tissue, A < B, $P = 0.014$; A > C, $P = 0.114$; A > D, $P = 0.005$; C > D, $P = 0.001$; B > D, $P = 0.000$, It also represented that the intensity of PTEN in medium dose group was highest ($P < 0.001$). However, there was no significant statistic difference between medium dose group and low group, low dose group and high dose group ($P > 0.05$) (Table 5).

**Table 5 Expression intensity of PTEN of 3 kinds of tissue of the 4 groups (mean ± SD)**

| Group | *n* | Normal hepatic tissue | Latero-cancer tissue | Cancer tissue (median) |
|---|---|---|---|---|
| A | 12 | 1.05 ± 0.34 | 1.42 ± 0.24 | 0.36 |
| B | 12 | 0.90 ± 0.31 | 1.75 ± 0.42 | 0.86 |
| C | 12 | 1.06 ± 0.18 | 1.27 ± 0.21 | 0.20 |
| D | 12 | 0.59 ± 0.48 | 0.64 ± 0.61 | 0.05 |

## DISCUSSION

PTEN/MMAC1 (mutated in multiple advanced cancer 1) or TEP1 (TGF-regulated and epithelial cell-riched phosphatase1), located in human chromosome band 10q23[11], was recently identified together as tumor suppressor gene by three America research teams. As the first discovered dual-specific phosphatase, it may suppress tumor cell growth, regulate tumor cell invasion and metastasis through inhibiting many signal pathways of cell proliferation[12-15].

FT-207/tegafur, which belongs to the Second-generation fluoropyrimidine drugs, is metabolized to 5-fluorouracil through certain hepatic metabolizing enzymes and the cytochrome P450 (CYP450) system. Its anticancer mechanism is the same with that of 5-FU[16]. The advantage of Tegafur is highly fat-soluble, rapid gastrointestinal absorption, much longer half-life, as well as suitable for oral administration, its side effects is only one seventh that of 5-FU, while the efficacy index is twice higher. As the representative of the Second-generation 5-FU, Tegafur is used to treat several types tumors of alimentary canal as a routine chemotherapy drugs[17-19].

TCM includes primary hepatic carcinoma in the category of diseases such as: liver mass, abdominal mass, ZhengJia, tympanites, jaundice, *etc.* The etiological factors are concerned with yin-yang disbalance caused by the reception humid heat, long-term eating and drinking without temperance, being addicted to drink, internal injury caused by excess of seven emotions. When the vital qi gets deficient, evil factor easily invades the body and are stagnated in the liver cause the depression of liver-QI, activities of qi is stagnated and the blood circulation is blocked; Phlegm knobbing stagnant blood leads to the formation of abdominal mass, finally resulting in the formation of liver cancer[20,21]. The primary pathogenesis of liver cancer lies in the qi-stagnancy and blood stasis, the stagnation of humid heat, the discord of the spleen and the liver, as well as the weakness of vital qi[22]. At the early stage, this disease manifests itself in the unimpaired vital-qi, the type is most likely be sthenia syndrome or syndrome of blood stasis; at the middle stage, vital vital-qi is impaired, and asthenia and sthenia complicated with each other; at the advanced stage, the main syndrome is asthenia syndrome. Thus, the main treatment of TCM for liver cancer lies in strengthening body resistance and eliminating pathogen; the former is the mian way, the later is the assistant way[23,24]. Correlated investigation confirmed that the therapy of invigorating the spleen and regulating the qi could inhibit or delay the tumorous growth and metastasis, strengthen body's immunity, prolong the life span, and was more effective than the therapy of promoting blood flow and dissolving the stagnated blood and of[25,26], what's more, different therapeutic methods of TCM can in different degree regulate the transcription of some important oncogenes which play an important role in the process of occurrence and development of the liver cancer[27], thereby have some antitumous effect. Therefore, TCM becomes one of important combined therapies for cancer in China. In dealing with tumor, Chinese crude drugs have such advantages as following: guidance of wholism, strengthening the internal anticancer mechanism, mild toxicity, without pain, easily be accepted, it can relieve the symptoms, improve patient's function, lighten the toxicity and side effect meanwhile enhance effectiveness of the radiotherapy or chemotherapy, and accelerate recovery from operation, further more, can inhibit tumor growth, control or delay its recurrence, improve life quality, prolong

survival time. During to the traditionary chemotherapy wih the characteristic of being exist without tumor impacts the patient's exist quality, while people pay close attention to TCM wirh the characteristic of being exist with tumor.

FJD (Appl.: 200710026976.6) has been widely used to treat the hepatocellular carcinoma for years in our department. According to the special pathogenesis of middle and advanced hepatocellular carcinoma, the decoction can smooth the liver and strengthen the spleen, meanwhile can strengthen the body resistance, remove toxin and treat Biaol and Ben. So the decoction has the effect of strengthening the spleen and replenishing qi, diminishing stagnation by detoxification, relieving the depressed liver qi and regulating the blood. Previous research showed that this decoction had a certain anticancer effect on liver cancer cell lines *in vitro*[28] and in clinic[29]. In this study, we found that each TCM groups had distinct superiority in prolonging the live time and raising the survival rate of athymic mouse bearing cancer compared with the chemotherapy group. At the same time, middle and high concentration groups were more effective in prolonging the live time than that in low concentration group, which indicated TCM groups in prolonging the live time had dose-effect relationship. However, the TCM groups and the chemotherapy group has the similar effect in shortening tumor. Moreover, each group of the medicinal herbs were better than chemotherapy group chemotherapy group in inhibiting tumor diffusion and reducing tumor metastasis in liver. Compared with chemotherapy group, every TCM groups could reduce the number of tumor metastasis in liver, there was no difference between TCM groups and chemotherapy group in tumor metastasis rate in liver, which might relate with the insufficient the number of the sample. As mentioned previously, FJD could inhibit tumor metastasis and prolong the live time, the antitumous effect of FJD might through certain way to inhibiting tumor diffusion and metastasis other than shorten tumor, thus slowed down pathogenetic condition progressing, and prolonged the live time[30].

What on earth is the mechanism of the anticancer treatment by the Chinese herbs that strengthen the body resistance and remove toxic substances? Our research showed that the low, middle and high concentrations of the Chinese herbs could obviously increase expression of PTEN on the athymic mice bearing tumor respectively, compared with chemotherapy by ft207, which indicated the antitumous mechanism of medicinal herbs in this study. Interestingly, we found the expression of the PTEN in adjacent cancerous tissues of athymic mice bearing tumor was higher than that of the normal tissues, we inferred that this phenomenon might be a protection of the body itself to prevent cancer cell from further developing by high expression of PTEN in adjacent cancerous tissues under stressful condition. Therefore, the expression density of PTEN closely relates with the occurrence and development of liver cancer. Our study showed that FJD can provocate the expression of PTEN, and high expression of the PTEN in adjacent cancerous tissues seem to explain the reason why the intrahepatic metastasis rate in TCM groups is lower than that of chemotherapy group. The comparison among the TCM groups indicated that middle concentration achieved the best curative effect, low and high groups ranked secondly, this result illustrates that it is necessary for us to pay more attention to the dosage of medicinal herbs in clinic practice, because only the moderate dosage may acquire the best therapeutic effect. However, the best concentration of the FJD in treating HCC needs to be studied furtherly.

However, a control group of mice which were inoculated HCCs in the liver and were not given FZDJT and tegafur had been allocated. It's a pity that due to the too much cellular necrosis in pathological section we cannot get the result of immunohistochemistry. Next time we should do better.

In conclusion, our study showed that FJD can prolong the survival time and decrease tumor metastasis in livers of these experimental mice. Mechanisms of FJD healing HCC may partially be explained by enhancing the expression of PTEN in liver.

## COMMENTS

### Background

Traditional Chinese Medicine (TCM) which is useful to improve life quality, prolong survival time has been widely used as combined therapies in treating hepatocellular carcinoma (HCC). The primary pathogenesis of liver cancer lies in the qi-stagnancy and blood stasis, the stagnation of humid heat, the discord of the spleen and the liver, as well as the weakness of vital qi. Fuzheng Jiedu Decoction (FJD) (Appl.: 200710026976.6) has been widely used to treat the hepatocellular carcinoma for years. Previous research showed that this decoction had a certain antitumous effect on liver cancer cell lines *in vitro* and in clinic.

### Research frontiers

TCM has been widely used as combined therapies in treating HCC in China. The novelty and innovation of the research consist in researching the mechanism of treatment of FJD to the hepatocellular carcinoma with mole-biological method. Mechanisms of FJD healing HCC may partially be explained by enhancing the expression of PTEN in liver.

### Innovations and breakthroughs

TCM has been widely used as combined therapies in treating HCC, whether it can down-regulate the expression of PTEN in HCC is still unknown. The aim of our study is to explore the effect of FJD, complex prescription of Chinese crude drug in treating BALB/c athymic mice with HCC, as well as the expression of PTEN.

### Applications

The mechanism of treatment of FJD to the hepatocellular carcinoma may partially be explained by enhancing the expression of PTEN tumor suppressor gene. We also found that different concentration of FJD had different effect in prolonging survival time and decreasing metastatic tumour. This study was indicated that FJD can be used as one of combined therapies in treating HCC and which was the best concentration of FJD.

### Terminology

PTEN: Phosphatase and tensin homolog deleted on chromosome ten. FJD: Fuzheng Jiedu Decoction. FT207: Tegafur. TCM :Traditional Chinese Medicine.

### Peer review

This is a good study in which the main objective is to explore the expression of PTEN in liver of athymic mice with HCC and the effect of FJD. PTEN was recently identified together as tumor suppressor gene. FJD can prolong the survival time and decrease Tumor metastasis in livers of these experimental mice. Mechanisms of FJD healing HCC may partially be explained by enhancing the expression of PTEN in liver.

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*RAPID COMMUNICATION*

# A clinical trial of combined use of rosiglitazone and 5-aminosalicylate for ulcerative colitis

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## Abstract

**AIM:** To investigate the therapeutic effects of the combined use of rosiglitazone and aminosalicylate on mild or moderately active ulcerative colitis (UC).

**METHODS:** According to the national guideline for diagnosis and treatment of inflammatory bowel disease (IBD) in China, patients with mild or moderately active UC in our hospital were selected from July to November, 2004. Patients with infectious colitis, amoebiasis, or cardiac, renal or hepatic failure and those who had received corticosteroid or immunosuppressant treatment within the last month were excluded. Following a quasi-randomization principle, patients were allocated alternatively into the treatment group (TG) with rosiglitazone 4 mg/d plus 5-ASA 2 g/d daily or the control group (CG) with 5-ASA 2 g/d alone, respectively, for 4 wk. Clinical changes were evaluated by Mayo scoring system and histological changes by Truelove-Richards' grading system at initial and final point of treatment.

**RESULTS:** Forty-two patients completed the trial, 21 each in TG and CG. The Mayo scores in TG at initial and final points were 5.87 (range: 4.29-7.43) and 1.86 (range: 1.03-2.69) and those in CG were 6.05 (range: 4.97-7.13) and 2.57 (range: 1.92-3.22) respectively. The decrements of Mayo scores were 4.01 in TG and 3.48 in CG, with a remission rate of 71.4% in TG and 57.1% in CG, respectively. Along with the improvement of disease activity index (DAI), the histological grade improvement was more significant in TG than in CG ($P < 0.05$).

**CONCLUSION:** Combined treatment with rosiglitazone and 5-ASA achieved better therapeutic effect than 5-ASA alone without any side effects. Rosiglitazone can alleviate colonic inflammation which hopefully becomes a novel agent for UC treatment.



**Key words:** Peroxisome proliferators-activated receptor γ; Rosiglitazone; Ulcerative colitis

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## INTRODUCTION

Ulcerative colitis (UC) is chronic intestinal inflammation with uneven relapsing course. Most patients need to take daily medications lifelong. The conventional therapies for UC include 5-aminosalicylate (5-ASA), glucocorticoid, and immunomodulator. 5-ASA and its derivatives were widely used to induce and maintain remission in patients with UC. But the general response rate was only 70%-80% and the relapse rate varied depending on the maintenance of the medications and other relapsing factors. It is necessary to search for special target of inflammatory cascades in UC so as to improve the therapeutic effects. With deeper understanding of its pathogenesis, more and more therapeutic targets have been found, and more and more novel clinical trials are directed to these targets with encouraging results. Peroxisome proliferators-activated receptor γ (PPAR-γ), highly expressed in the colon, is a subgroup of ligand-activated nuclear receptors responsible for the regulation of cellular events ranging from lipid homeostasis to cell differentiation and apoptosis. Recent studies showed its ligands can suppress the inflammatory response by inhibiting the activity of macrophages, cytokines production and NF-κB transcriptional activities. Thiazolidinediones (TZDs), such as rosiglitazon, are PPAR-γ synthetic ligands which have been used in type 2 diabetes mellitus for a long time. James *et al*[1] reported that refractory UC patients achieved clinical and endoscopic remissions using rosiglitazone, and concluded that it might represent a novel approach of UC therapy. However, it is an open-label trial with a small sample size and needs to be validated by expanded trials.

In this study we observed the therapeutic effects of combined use of rosiglitazone and 5-ASA in UC clinically and improvement pathologically.

## MATERIALS AND METHODS

### Design

Patients with mild or moderately active UC, who met with the criteria of suggested guidelines for the diagnosis and treatment of inflammatory bowel disease (IBD), were enrolled[2]. Patients with infectious colitis, acute or chronic cardiac, renal and hepatic failure and abnormal liver-associated chemistries were excluded. Patients who took glucocorticoid or immunomodulators, such as AZA, 6-MP, within the last month were also excluded. Included patients all provided informed consent. During the trial, the patients were not allowed to take traditional Chinese medicines (TCM), glucocorticoid and immunomodulator.

### Assignment

The patients were assigned into treatment group (TG) or control group (CG), with even number allocated into TG and odd number into CG. Both groups took 5-ASA 2 g/d (500 mg granule, Beaufour Ipsen Pharmaceutical Co. Ltd), but rosiglitazone 4 mg/d (4 mg/tablet, GlaxoSmithKline Pharmaceutical Co. Ltd.) was added to the treatment group for 4 wk.

### Follow-up

The patients were assessed from 0 to the 4$^{th}$ wk and interviewed by phone every week and returned for a visit at the 2$^{nd}$ wk, and received the colonoscopic examination and colonic mucosal sampling at initial and final points. The evaluation of disease activity was performed at 0 and the 4$^{th}$ wk according to the Mayo indices[3] (also called Sutherland index, Table 1).

**Table 1 Mayo DAI indices**

| | |
|---|---|
| Stool frequency | |
| 0 | Normal number of stools for this patient |
| 1 | 1 to 2 more stools than normal |
| 2 | 3 to 4 more stools than normal |
| 3 | 5 or more stools than normal |
| Rectal bleeding | |
| 0 | No blood seen |
| 1 | Streaks of blood with stool less than half the time |
| 2 | Obvious blood with stool most of the time |
| 3 | Blood alone passed |
| Findings of endoscope | |
| 0 | Normal or inactive disease |
| 1 | Mild disease (erythema, decreased vascular pattern, mild friability) |
| 2 | Moderate disease (marked erythema, absent vascular pattern, friability, erosions) |
| 3 | Severe disease (spontaneous bleeding, ulceration) |
| Physician's global assessment | |
| 0 | Normal |
| 1 | Mild disease |
| 2 | Moderate disease |
| 3 | Severe disease |

### Definition of outcome

Patients with a final DAI score of $\leqslant 2$ were defined to achieve clinical remission and patients with a final score $\geqslant 3$ but reduction in the DAI of $\geqslant 2$ were defined to achieve partial remission.

### Safety analysis

All patients were included in the safety analysis. The adverse events and concomitant medication were carefully documented. Safety evaluations included vital signs, patients' symptoms, physical examination, hematology, serum biochemistry, fecal routine, and urinalysis. All the patients received these tests at the initial and the final points of the study.

### Histopathology

At least 2 colon samples were taken from each patient at initial and final points of the trial through colonoscopy. Tissues were fixed in 10% buffered neutral formalin and embedded in paraffin. Four μm sections were stained with the hematoxylin and eosin (HE stain) for histological evaluation. Tissue slides were blindly assessed by experienced pathologists based on the Truelove-Richards histological grading system[4] as follows: 0 = no polymorphs, 1 = small number of polymorphs in the lamina propria with minimal infiltration of crypts, 2 = prominent polymorphs in the lamina propria with infiltration of > 50% of crypts, 3 = florid polymorph infiltrate with crypt abscesses, and 4 = florid acute inflammation with ulceration.

### Immunohistochemistry

Labeled streptavidin-biotin (LsAB) methods were used for PPAR-γ and NF-κB p65 detection. Paraffin-embedded colonic tissue samples were dewaxed in xylene for 5 min twice, rehydrated in a series of ethanol (100%-70%) for 3 min each followed by rehydration in PBS for 30 min. After rehydration, the endogenous peroxidase was blocked with 0.3% hydrogen peroxide followed by antigen retrieval by autoclaving sections in citrate buffer pH 6.0 (10 mmol/L Na citrate). After antigen retrieval, the sections were stained using the above-mentioned kit according to manufacturer's recommendations, but with the following modifications. Sections were incubated with the primary antibody at 37℃ for 2 h. The following antibodies were used at the indicated dilutions: PPAR-γ (E-8: sc-7273, Santa Cruz, Santa Cruz, CA USA) 1:100 and NF-κB p65 (Boshide Bio Corp, Wuhan, China) 1:200. Each section had its own control using the secondary antibody only. Preimmune serum was initially used to ensure specificity of the signal with each of the antibodies. The lipoma resection sample was used as the positive control and the substitute monoclonal antibody of PPAR-γ with PBS as the blank control.

### Statistical analysis

Statistical analysis was performed with the SPSS 10.0 software. The *t* test of the sample mean value of the designed group was used to examine the Mayo scores and positive cell numbers in the immunohistochemistry. The rank sum test was adopted for the histological grading. A $P$ value $< 0.05$ was considered statistically significant.

## RESULTS

### Patient characteristics

We enrolled 42 patients with mild and moderately active UC into our trial and 21 patients each in TG and CG.

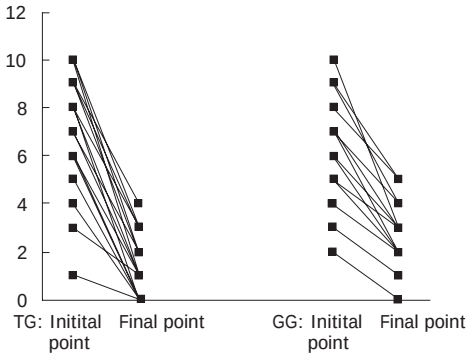


**Figure 1** Mayo scores of TG and CG at initial and final points.

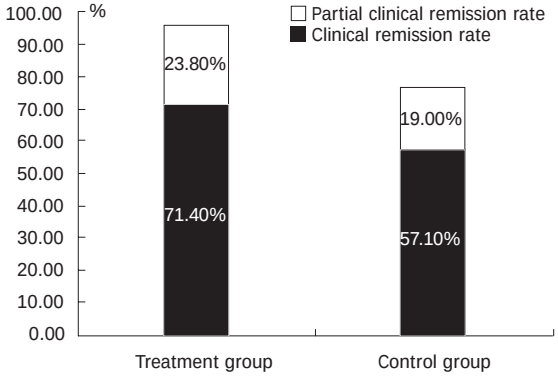


**Figure 2** The clinical remission rate of TG *vs* CG.

The pre-study characteristics of patients are summarized in Table 2. The age ranged from 16 to 57 years (average, 38.8 ± 10.4 years) in TG and from 14 to 60 years (average, 37.1 ± 8.01 years) in CG, respectively. The extent of disease in TG was 8 proctitis (38.1%), 12 proctosigmoiditis (57.1%), and 1 left-side colitis (4.8%) and 9 (42.9%), 11 (52.4%), 1 (4.7%) in CG respectively. All patients received 5-ASA agents before enrolled into this study. The statistical analysis on the age, sex, location, type, and disease activity suggested similar baseline characteristics in the two groups ($P > 0.05$).

### *DAI scores at initial and final points*

The Mayo score changes in each patient from 0 to the end of 4 wk were figured as below (Figure 1): the DAI in TG at initial and final points were 5.87 (range, 4.29-7.43) and 1.86 (range, 1.03-2.69), respectively, and those in CG were 6.05 (range, 4.97-7.13) and 2.57 (range, 1.92-3.22) respectively, (Table 3). There was a significant difference between the two groups and the therapeutic effect of TG was better than CG ($P < 0.05$).

According to the definition of outcome from the guideline, the clinical remission was achieved in 15 patients in TG (remission rate, 71.4%) and 12 patients in CG (remission rate, 57.1%), the partial remission was achieved in 5 patients (23.8%) and 4 patients (19.0%) in TG and CG respectively (Figure 2).

### *Histological grade at initial and final points*

According to Truelove and Richards's histological grading system, the histological sections by H&E staining were graded in Table 4. The grade Ⅱ and Ⅲ of histology was found at the initial point (80.95%) and grade Ⅰ (57.14%) at the final points in TG, respectively, as compared with 33% grade Ⅰ at the final point in CG.

### *Immunohistochemistrical positive percentages at initial and final points*

The positive percentage of PPAR-γ at initial and final points were 26.20%, 47.76% in TG and 24.90%, 39.24% in CG, respectively. The positive percentage of NF-κB were 58.09%, 21.19% and 58.00%, 27.00%, accordingly (Table 5). The relation between histological grade and immunohistochemistrical positive percentages was showed on Table 6.

**Table 2 Clinical characteristics of ulcerative colitis**

| | | TG ($n$ = 21) | CG ($n$ = 21) |
|---|---|---|---|
| Age (yr) | | 38.8 ± 10.4 | 37.1 ± 8.01 |
| Sex | Male (%) | 16 (76.2) | 15 (71.4) |
| | Female (%) | 5 (23.8) | 6 (28.6) |
| Location | Rectum (%) | 8 (38.1) | 9 (42.9) |
| | Rectosigmoid (%) | 12 (57.1) | 11 (52.4) |
| | Left colon (%) | 1 (4.8) | 1 (4.7) |
| Type | Chronic relapse (%) | 13 (61.9) | 12 (57.1) |
| | Chronic persistence (%) | 8 (38.1) | 9 (42.9) |
| Severity | Mild (%) | 5 (23.8) | 6 (28.6) |
| | Moderate (%) | 16 (76.2) | 15 (71.4) |

**Table 3 Mayo scores of TG and CG at initial and final points**

| Mayo scores | Initial point | Final point |
|---|---|---|
| TG ($n$ = 21)[a] | 5.87 (4.29-7.43) | 1.86 (1.03-2.69) |
| CG ($n$ = 21) | 6.05 (4.97-7.13) | 2.57 (1.92-3.22) |

[a]$P < 0.05$ *vs* CG.

**Table 4 Histological grade of TG and CG**

| Grade | TG ($n$ = 21) | | CG ($n$ = 21) | |
|---|---|---|---|---|
| | Initial point | Final point | Initial point | Final point |
| Ⅰ[a] | 4 | 12 | 5 | 8 |
| Ⅱ[c] | 10 | 6 | 12 | 11 |
| Ⅲ[e] | 7 | 3 | 4 | 2 |
| Ⅳ | 0 | 0 | 0 | 0 |

[a]$P < 0.05$, TG *vs* CG; [c]$P < 0.05$, TG *vs* CG; [e]$P < 0.05$, TG *vs* CG.

**Table 5 Positive percentages of PPAR-γ and NF-κB in TG and CG**

| | TG ($n$ = 21) | | CG ($n$ = 21) | |
|---|---|---|---|---|
| | Initial point | Final point | Initial point | Final point |
| PPAR-γ[a] (%) | 26.2 | 47.76 | 24.9 | 39.24 |
| | (22.98-29.49) | (43.42-50.09) | (21.49-28.32) | (35.81-42.67) |
| NF-κB[c] (%) | 58.09 | 21.19 | 58 | 27 |
| | (54.51-61.68) | (19.13-23.25) | (53.21-61.02) | (21.08-32.92) |

[a]$P < 0.05$, initial point to final point of TG *vs* CG; [c]$P < 0.05$, initial point to final point of TG *vs* CG.

**Table 6 Histological grade and PPAR-γ and NF-κB expression**

| | | Expression positivity (100%) | | | |
|---|---|---|---|---|---|
| | | Ⅰ | Ⅱ | Ⅲ | Ⅳ |
| Initial points | *n* | 4 | 10 | 7 | 0 |
| | NF-κB | 46.09-51.91 | 52.11-56.89 | 61.35-70.65 | 0 |
| | PPAR-γ | 31.57-39.93 | 24.54-29.06 | 16.30-20.55 | 0 |
| Final points | *n* | 12 | 6 | 3 | 0 |
| | NF-κB | 21.55-26.62 | 18.93-20.07 | 8.42-20.92 | 0 |
| | PPAR-γ | 37.20-44.97 | 49.82-56.51 | 55.50-69.84 | 0 |

### *Safety among treatment group and control group*

During the observation period, no adverse events and abnormalities in the blood biochemical test were found in the patients. No jaundice or edema was found either.

## DISCUSSION

As it is known, except for open-label trial by James *et al*[1], there are few clinical trials about rosiglitazone as adjunctive agent for UC conventional therapy. In this trail, treatment group showed a greater decrease in DAI and higher clinical remission rate than control group. Our results showed similar effects as those of James and his colleagues and the adjunctive effect of rosiglitazone on the UC therapy. However, we investigated the effects of rosiglitazone in mild and moderately active UC, but James did in refractory UC.

The PPAR-γ, highly expressed on adipo-tissue and colons, can regulate gene transcription of lipoprotein, cell differentiation, inflammation and immune response. PPAR-γ plays a key role not only in adipocyte differentiation, insulin sensitivity and nonalcoholic fatty liver[6-10], but also in innate and adaptive immunity[11-13]. Activation of PPAR-γ can promote macrophage desensitization, thus attenuating the oxidative burst[14]. Arnold and Konig[15] observed that PPAR γ ligands inhibited dose-dependently the release of TNF-α, GM-CSF, IL-1α, IL-6, IL-8 and CCL5 from RSV-infected A549 cells while diminishing the cellular amount of mRNA of IL-6, IL-8 and CCL5 and binding activity of the transcription factors NF-κB and AP-1, respectively. Belvisi *et al*[16] also outlined the anti-inflammatory effects of PPAR-γ ligands. Pan *et al*[17] revealed this anti-inflammation effect on human gallbladder epithelial cells as well. PPAR-γ ligands can prevent intestinal inflammation by blocking the activation of NF-κB, down-regulate the production of ICAM-1 and TNF-α in intestinal epitheliums, suppress expressions of TNF-α and IL-1β, *etc.*

Dubuquoy *et al*[5] found that expression of PPAR-γ in colon mucosa was decreased in active UC and was negatively related with UC severity. PPAR-γ was regarded as a new therapeutic target in IBD, especially in UC[18]. Christel *et al*[19] even found that the intestinal anti-inflammatory effect of 5-ASA depends on the PPAR-γ in chemically induced colitis in mice heterozygous at the PPAR-γ locus. These results strongly suggested a reasonable combination of these two agents for the therapeutic purpose on UC.

There are two kinds of PPAR-γ ligands, one is natural ligands such as prostaglandins J2, the other is synthetic ligands including TZDs. TZDs (e.g. rosiglitazone and pioglitazone) can reduce the productions of several pro-inflammatory cytokines and ameliorate the intestinal inflammation. Adachi M *et al*[20] reported that PPAR-γ in colonic epithelial cells plays an anti-inflammatory role and protects against experimental IBD. Marina Sánchez-Hidalgo *et al*[21] draws a similar conclusion. Sasaki *et al*[22] reported that troglitazone significantly reduced the TNF-α mediated induction of endothelial MAdCAM-1 in a dose-dependent manner; it also lowered the VCAM-1, ICAM-1 and E-selectin expression and significantly reduced α4β7-integrin dependent lymphocyte adhesion. Bassaganya *et al*[23] found that activation of PPAR-γ can inhibit the activation of NF-κB and ameliorate experimental colitis. NF-κB controls the transcription of a large cohort of genes, its dysregulated activation is linked to various biological disorders including inflammatory, and immune disorders[24]. In IBD, NF-κB plays an important role and is a target of various anti-inflammatory drugs. The ability of TZDs to ameliorate the experimental colitis is closely related to inhibition of NF-κB activation[19]. Another unpublished study of ours also investigated colonic mucosal expressions of PPAR-γ and NF-κB in the oxalzolone-induced experimental colitis, and found that the PPAR-γ ligand could increase the expression of PPAR-γ and inhibit the activation of NF-κB in colonic epithelium, ameliorating the colon inflammation in experimental colitis. Lawrence *et al*[25] showed that PPAR-γ ligands could provide anti-inflammatory protection by maintaining the cytokine balance and shifting transcriptional regulation of T cells away from Th1 and towards Th2 predominance in acute DSS colitis. Lytle *et al*[26] found that rosiglitazone could slow down the onset of spontaneous IBD in IL-10 (-/-) mice. Sánchez-Hidalgo *et al*[27] also reported that rosiglitazone for TNBS colitis can correct mucosal lesions, and significantly lower the ulceration index, myeloperoxidase (MPO), and the levels of TNF-α. Meanwhile, it increased prostaglandin (PG) $E_2$ production and returned PG $D_2$ to basal levels and reduced COX-2 and NF-κB proteins expression. Pedersen *et al*[28] reported that PPAR-γ mRNA and adipophilin expressions (a marker of PPAR activation) were markedly lower in colonic epithelium cells (CEC) from the patients with active UC and were clearly associated with the inhibition of PPAR-γ function and the stimulation with rosiglitazone fully restored PPAR-γ activation in CEC. Zhang YQ *et al*[29] reported that rosiglitazone enhances apoptosis of HT-29 cells by activating PPAR-γ. Li *et al*[30] reported troglitazone can not inhibit cell proliferation, and induce apoptosis in HepG2 cells, but down-regulate the expression of COX-2 mRNA and protein.

Our study showed the therapeutic effects of rosiglitazone on human UC, but it is required to demonstrate whether the therapeutic effects of rosiglitazone or other PPAR-γ ligands depend on PPAR-γ NF-κB signal pathway or on other inflammatory pathway, such as P38 MAPK, or toll-like receptor. A larger sample size clinical trail is also needed to confirm the effects of rosiglitazone on human UC.

Rosiglitazone has few adverse effects except for an ALT increase and exacerbation of cardiac failure induced

by the body fluid retention. In our study, we excluded the patients with hepatic, cardiac failure and closely observed related clinical and chemical changes. No adverse events were found in our study, including 4 patients who took rosiglitazone for 12 wk. Based on the above observation, we conclude that TZDs, ligands of PPAR-γ can be used in the UC as an adjunctive agent efficiently and safely.

## COMMENTS

### Background

The peroxisome proliferators activated receptor γ (PPAR-γ) is highly expressed in the colon and play a crucial role in intestinal inflammation. Regulation of colon inflammation by PPAR-γ has been well demonstrated in experimental colitis. Recently, rosiglitazone achieved quite well therapeutic effects in refractory ulcerative colitis (UC) patients in the study of James D. This result suggested that PPAR-γ is a hopefully novel target for UC treatment in the future.

### Research frontiers

The hotspots in this field of studies include mechanism of PPAR-γ in intestinal inflammation and finding its high-affinity ligands.

### Innovations and breakthroughs

An open-label trial of rosiglitazone for UC by James D *et al* was a first research of clinical application of thiazolidinediones (TZDs); however, their study merely focused on refractory UC. We mainly studied the patients with active UC and explored the mechanisms of PPAR-γ ligands.

### Applications

PPAR-γ has multiple functions in the immune system and in some major inflammatory diseases such as atherosclerosis, inflammatory bowel disease and rheumatoid arthritis. If the ligands of PPAR-γ can be utilized for UC treatment, it will provide another therapeutic target and improve the effects of UC treatment.

### Terminology

PPAR-γ is a member of nuclear receptors family, proved to be a key transcription factor of adipocyte differentiation, lipid and glucose homeostasis and an important target in type 2 diabetes and metabolic syndrome. Besides its role in metabolic tissues, it appears to be expressed in several other cells, including immune cells such as macrophages, dendritic cells, eosinophils, T cells and B cells. It was pointed to a role in the immune system and its new aspect was revealed and developed in parallel: its potential anti-inflammatory activity. DAI is an abbreviation of disease activity index and widely used to evaluate the conditions and therapeutic effect for UC. There are several DAI systems; however, Mayo system is widely accepted.

### Peer review

The authors aim to investigate the therapeutic effects of rosiglitazone on mild or moderately active ulcerative colitis and explore the relation between PPAR-γ and intestinal inflammation and NF-κB. Their results revealed the ligand of PPAR-γ can alleviate the inflammation of UC and this effect may be related with depression of NF-κB by PPAR-γ activation. Rosiglitazone can alleviate colonic inflammation and hopefully become a novel agent for the treatment of UC. PPAR-γ may be another therapeutic target of UC in the future.

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*RAPID COMMUNICATION*

# Comparison of ligase detection reaction and real-time PCR for detection of low abundant YMDD mutants in patients with chronic hepatitis B

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## Abstract

**AIM:** To compare the ligase detection reaction (LDR) and real-time PCR for detection of low abundant YMDD mutants in patients with chronic hepatitis B infection.

**METHODS:** Mixtures of plasmids and serum samples from 52 chronic hepatitis B patients with low abundant lamivudine-resistant mutations were tested with LDR and real-time PCR. Time required and reagent cost for both assays were evaluated.

**RESULTS:** Real-time PCR detected 100, 50, 10, 1 and 0.1% of YIDD plasmid, whereas LDR detected 100, 50, 10, 1, 0.1, and 0.01% of YIDD plasmid, in mixtures with YMDD plasmid of $10^6$ copies/mL. Among the 52 clinical serum samples, completely concordant results were obtained for all samples by both assays, and 39 YIDD, 9 YVDD, and 4 YIDD/YVDD were detected. Cost and time required for LDR and real-time PCR are 60/80 CNY (8/10.7 US dollars) and 4.5/2.5 h, respectively.

**CONCLUSION:** LDR and real-time PCR are both sensitive and inexpensive methods for monitoring low abundant YMDD mutants during lamivudine therapy in patients with chronic hepatitis B. LDR is more sensitive and less expensive, while real-time PCR is more rapid.



**Key words:** YMDD mutants; Hepatitis B virus; Real-time PCR; Ligase detection reaction

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## INTRODUCTION

Lamivudine is an effective antiviral agent for treatment of patients with chronic hepatitis B and advanced liver diseases[1]. However, long-term lamivudine monotherapy leads to emergence of lamivudine-resistant hepatitis B virus (HBV) mutants in some patients chronically infected with HBV[1,2]. The incidence is 16%-32% in the first year and increases to 38%, 57%, and 67% after 2, 3, and 4 years, respectively[3-6]. Resistance is associated with mutations in the highly conserved tyrosine-methionine-aspartate-aspartate (YMDD) motif of the reverse transcriptase, which is part of the catalytic site of the HBV polymerase[7]. Viological breakthrough and alanine transaminase (ALT) elevation have been shown to occur 2-28 wk and 12-31 wk after the emergence of YMDD mutants, respectively[8-10]. Initially, YMDD mutants consist of minor populations. They gradually replace the wild-type virus, reaching a 100% lamivudine-resistant variant population, and this replacement occurs in parallel with the increase in HBV DNA load[10]. Sensitive methods for early detection of lamivudine-resistant mutants will be helpful for physicians to make clinical decisions in treatment of patients with HBV infection.

Several technologies have been developed for the detection of lamivudine-resistant mutants[11]. Although nucleotide sequencing of PCR products is widely used to detect lamivudine resistance, it is expensive and laborious, and can detect only mutant viruses representing at least 50% of the total virus population[8]. Inno-LiPA, pyrosequencing, real-time PCR, and ligase detection reaction (LDR) are able to detect low abundant YMDD mutants in the wild-type HBV[8,12-15]. However, only few studies have compared these methods.

We have previously compared real-time PCR and pyrosequencing for detection of YMDD mutants in patients with chronic hepatitis B[16]. In the present study, we compared LDR and real-time PCR for detection of low abundant YMDD mutants in mixed plasmids and clinical

samples from lamivudine treated patients with chronic hepatitis B.

## MATERIALS AND METHODS

### Plasmids and controls

Plasmids and controls were prepared as previously described[14,16]. In brief, three previously identified serum samples containing HBV with YMDD, YVDD and YIDD sequences were used as template and amplified by PCR. PCR products were cloned using pGEM-T systems (Promega, Madison, Wisconsin, USA), and clones were sequenced using ABI 3100 sequencer (Applied Biosystems, Foster, California, USA).

### Patients and samples

Serum samples were collected from 196 patients with chronic HBV infection. All patients were treated with lamivudine for three months to three years and serum HBV-DNA levels were above $1.0 \times 10^4$ copies/mL by real-time PCR. Among these samples, 52 samples with YMDD mutants below 50% of total HBV population (determined by real-time PCR[14]) were selected for comparison of LDR and real-time PCR. All these 52 samples were found to contain only the YMDD variant by sequencing of PCR products, but found to contain YVDD or YIDD variants with real-time PCR or LDR.

### Extraction and quantitation of HBV DNA

HBV DNA was extracted from serum samples using the HBV DNA extraction reagents (Fosun Diagnostics, Shanghai, China) according to the manufacturer's instructions. Serum HBV DNA levels were measured on ABI 7300 real-time PCR system (Applied Biosystems, Foster, California, USA) with quantitative real-time PCR reagents (Fosun Diagnostics, Shanghai, China), which was approved by the State Food and Drug Administration of China for *in vitro* diagnostic use.

### Sequencing of PCR products

HBV DNA samples were prepared for sequencing by amplification with PCR as described by Allen *et al*[7]. HBV DNA extracted from serum samples was amplified by PCR. PCR products were purified with QIAquick PCR purification kits (Qiagen, Chatsworth, California, USA) and were eluted from the column with 80 μL of distilled deionized water. The DNA quality and concentration were determined by absorbance measurements at 260 and 280 nm and by gel electrophoresis on a 2.5% agarose gel. All sequencing reactions were performed on ABI 3130 DNA sequencer (Applied Biosystems, Foster, California, USA).

### Ligase detection reaction

LDR was carried out as described by Xiao *et al*[15]. In brief, for one type of mutant (YIDD or YVDD), one common probe and two discriminating probes for mutant and wild-type YMDD were used in LDR, which was carried out in 20 μL of buffer, 1 pmol of each probe, and 5 μL of sample DNA. The reaction mixture was incubated at 94℃ for 2 min, before adding 15 U of thermostable Taq DNA ligase (New England Biolabs, USA), followed by 20 cycles of 30 s at 94℃ and 4 min at 65℃. Two PCR reactions were performed with the product of the LDR as template for 30 cycles at 94℃ for 30 s, 60℃ for 30 s, and 72℃ for 45 s. The PCR products were separated by agarose gel electrophoresis and visualized with ethidium bromide staining.

### Real-time PCR

Real-time PCR for detection of YMDD mutants was performed as previously described[14,16]. In brief, parallel reaction C, V and I were used to detect total HBV, YVDD and YIDD variants, respectively. The amplification was performed on ABI 7300 PCR system (Applied Biosystems, Foster, CA, USA) by incubating the reaction mixture (50 μL) at 50 degree for two minutes, followed by 5 min at 95 degree, 40 cycles of PCR amplification (94 degree for 20 s and 53 degree for 30 s). The reaction system was provided and optimized by Fosun Diagnostics (Fosun Diagnostics, Shanghai, China). The percentage of mutants in total virus was calculated by the following equations[14]:

(1) $\Delta Ct$ = Ct of control - Ct of mutants

(2) Ratio of mutants to total virus = $2^{\otimes X\tau}$

### Mixing experiments

Mixing experiments were carried out as previously described[16]. In brief, mutant plasmid containing YIDD sequence and wild-type plasmid were mixed at a final concentration of $10^6$ copies/mL, and the percentage of the YIDD plasmid in the mixture was 100%, 50%, 10%, 1%, 0.1%, and 0.01%, respectively. The mixtures were analyzed by LDR and real-time PCR respectively. For real-time PCR, each mixture was analyzed five times, and the mean Ct value of the five runs was used to determine the ratio of mutant to total viruses. For LDR assay, each mixture was analyzed only once.

### Time study

Two skilled technicians were selected to perform the assays. Time required for each assay was measured by direct observation during the procedures performed by the technicians, including the process of DNA extraction, amplification, detection, and analysis.

### Cost analysis

Cost for each assay was estimated based on the prices of reagents in China. The cost of instruments and labors was not included.

## RESULTS

### Detection of mixed plasmids

Mixtures of plasmids contained YIDD and YMDD at different ratios were detected by LDR and real-time PCR, respectively. LDR detected YIDD, YIDD/YMDD in the mixtures containing 100%, 50%, 10%, 1%, 0.1% and 0.01% YIDD plasmid. Real-time PCR detected YIDD in the mixture containing 100% YIDD plasmid and YIDD/YMDD in the mixtures containing 50%, 10%, 1% and 0.1% YIDD plasmids, but detected only YMDD in the mixture

**Table 1 Results of LDR and real-time PCR for detection of mixed plasmids containing YIDD and YMDD at a final concentration of $10^6$ copies/mL**

| YIDD plasmid in the mixture | LDR | Real-time PCR |
|---|---|---|
| 100% ($10^6$ copies/mL) | YIDD | YIDD |
| 50% ($5 \times 10^5$ copies/mL) | YIDD/YMDD | YIDD/YMDD |
| 10% ($10^5$ copies/mL) | YIDD/YMDD | YIDD/YMDD |
| 1% ($10^4$ copies/mL) | YIDD/YMDD | YIDD/YMDD |
| 0.1% (1000 copies/mL) | YIDD/YMDD | YIDD/YMDD |
| 0.01% (100 copies/mL) | YIDD/YMDD | YMDD |

containing 0.01% YIDD plasmid (Table 1). It means that real-time PCR and LDR are able to detect 1000 and 100 copies/mL of mutant virus in the background of wild type viruses, respectively. The results of real-time PCR were consistent with our previous study with mixtures of YVDD and YMDD plasmids[16].

### *Comparison of LDR and real-time PCR for detection of clinical samples with low abundant YMDD mutants*

We tested clinical serum samples from 52 lamivudine treated patients with chronic hepatitis B who had low abundant YMDD mutants. All the samples were detected as YMDD virus by sequencing the PCR products. The results obtained by LDR and real-time PCR were consistent (Table 2). Both methods detected 39 YIDD, 9 YVDD, and 4 YIDD/YVDD. The percentages of mutants in the virus population obtained by real-time PCR ranged from 4% to 40%. The percentage of the four YIDD/YVDD mixed mutants was 10%/20%, 30%/20%, 40%/10%, and 20%/30%, respectively.

### *Time required*

In this study, we used 96-well PCR equipment and all the 52 samples were dealt with in a run. The total assay time for LDR and real-time PCR was 4.5 and 2.5 h, respectively.

### *Cost*

The cost per test for each assay was calculated based on the prices of the reagents in China. Primers and probe were synthesized in TaKaRa Biotech (TaKaRa, Dalian, China). Real-time PCR mixtures were from Fosun Diagnostics (Fosun Diagnostics, Shanghai, China). The total reagent cost per test for LDR and real-time PCR was 60 and 80 CNY (8 and 10.7 US dollars), respectively. Although the cost of labors is similar in the same region, the cost of equipment used for LDR assay is much lower than that for PCR assay (5000 US dollars *vs* 60 000 US dollars).

## DISCUSSION

Lamivudine has revolutionized the treatment of chronic hepatitis B. Lamivudine-resistant mutations in the YMDD motif of polymerase gene were detected in lamivudine treated and untreated patients with chronic hepatitis B[14,17-19]. Clinical breakthrough was observed 2 wk-7 mo after the emergence of YMDD mutations[8-10], causing considerable morbidity and mortality in those patients[20-24].

**Table 2 Comparison of results obtained by LDR and real-time PCR for 52 clinical samples**

| Types | No. of samples (%) | |
|---|---|---|
| | LDR | Real-time PCR |
| YMDD | 0 (0) | 0 (0) |
| YIDD | 39 (75) | 39 (75) |
| YVDD | 9 (17) | 9 (17) |
| YIDD + YVDD | 4 (8) | 4 (8) |

Lamivudine-resistant mutants are frequently preexisting variants in HBV-infected patients and are selected during lamivudine therapy. These resistant variants initially represent a minority of the quasispecies and gradually replace the wild-type YMDD variants[10]. Detection of low abundant lamivudine-resistant mutants in the background of wild-type HBV as early as possible is helpful for virological follow-up and diagnosis of resistance in the clinical setting.

To date, many assays have been used for detection of lamivudine-resistant mutants in patients with hepatitis B[11]. The differences in sensitivity, specificity, cost, and time required do exist in these methods. Real-time PCR is able to quantitatively detect a small portion of resistant mutants in HBV populations and LDR is a newly developed method for detection of low abundant mutants in the background of wild-type HBV. In the present study, we compared LDR and real-time PCR for detection of low abundant YMDD mutations in lamivudine treated patients. The results obtained by the two methods were completely concordant in all samples, and 39 YIDD, 9 YVDD, and 4 YIDD/YVDD variants were detected. The percentages of mutants in the virus population obtained by real-time PCR ranged from 4% to 40%. In the mixing experiment, LDR was able to detect as low as 0.01% (100 copies/mL) of YIDD plasmid, while real-time PCR only detected 0.1% (1000 copies/mL) of YIDD plasmid in the background of YMDD plasmid. This may be due to LDR employing two kinds of amplification cycles, 20 cycles of LDR and 30 cycles of PCR, in the testing process. These results suggest that LDR is more sensitive than real-time PCR. In addition, the cost of LDR is slightly lower than that of real-time PCR. However, real-time PCR is much more rapid and requires less manual work than LDR. Both methods are sensitive and inexpensive compared to other methods for detection of YMDD mutation[16]. Another advantage of the real-time PCR method is that it is able to calculate the ratio of mutants to total virus in samples[14]. This will be useful in the clinical studies on the dynamics of resistant mutants during lamivudine therapy.

Several antiviral agents, such as adefovir and entecavir, can provide effective therapies in patients with lamivudine-resistant HBV[25-27]. Pegylated interferon also induces sustained responses in a portion of lamivudine-resistant patients[28-30]. Monitoring low abundant YMDD mutation during lamivudine therapy by sensitive and inexpensive methods will be helpful for physicians to make better clinical decisions as early as possible in management of chronic hepatitis B.

In conclusion, both LDR and real-time PCR are sensitive and inexpensive methods for monitoring low abundant YMDD mutations during lamivudine therapy in patients with chronic hepatitis B. LDR is more sensitive and less expensive, while real-time PCR is more rapid.

## COMMENTS

### *Background*

Many assays have been used for detection of lamivudine-resistant mutants in patients with hepatitis B. The differences in sensitivity, specificity, and cost do exist in these methods. However, only a few studies have compared these methods.

### *Research frontier*

Lamivudine-resistant variants initially represent a minority of the viruses and gradually replace the wild-type YMDD variants. Methods for detection of low abundant lamivudine-resistant mutants in the background of wild-type hepatitis B virus (HBV) as early as possible are helpful for diagnosis of resistance in the clinical setting.

### *Related publications*

Shi *et al* and Xiao *et al* developed real-time PCR and LDR assays for detection of minority lamivudine-resistant mutants in patients with hepatitis B. However, they did not compare the clinical performance between the two methods.

### *Innovations and breakthroughs*

This article compared LDR and real-time PCR for detection of low abundant YMDD mutations in lamivudine treated patients. Both assays are sensitive and inexpensive for monitoring low abundant YMDD mutations during lamivudine therapy in patients with chronic hepatitis B. LDR is more sensitive and less expensive, while real-time PCR is more rapid.

### *Applications*

Both LDR and real-time PCR are suitable for early detection of lamivudine-resistant mutations in patients treated with lamivudine.

### *Terminology*

Ligase detection reaction (LDR) detects nucleotide sequence by annealing and subsequent ligation of two oligonucleotides (probe and detector). Ligation of the probe and detector occurs only when the two bases on either side of the ligation site are complementary to the template. LDR is usually coupled with PCR for detection of low abundant point mutations.

### *Peer review*

This study is of importance for the early detection of lamivudine-resistant HBV mutants in patients with chronic HBV infection. The experiments appear to be conducted very carefully and by an experienced team of investigators.

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*CASE REPORT*

# Intermittent gastric outlet obstruction due to a gallstone migrated through a cholecysto-gastric fistula: A new variant of "Bouveret's syndrome"

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## Abstract

Bouveret's syndrome, defined as gastric outlet obstruction due to a large gallstone, is still one of the most dramatic biliary gallstone complications. Although new radiological and endoscopic techniques have made pre-surgical diagnosis possible in most cases and the death rate has dropped dramatically, "one-stage surgery" (biliary surgery carried out at the same time as the removal of the gut obstruction) should be still considered as the gold standard for the treatment of gallstone ileus. In this case, partial gastric outlet obstruction resulted in an atypical and insidious clinical presentation that allowed us to perform the conventional one-stage laparatomic procedure that completely solved the problem, thus avoiding any further complications.



**Key words:** Bouveret's Syndrome; Biliary gallstone; Gastric outlet obstruction; Biliary surgery

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## INTRODUCTION

Gallstones are completely asymptomatic in the majority of patients (60%-80%)[1]. When they become symptomatic, biliary colic is usually the first manifestation to be encountered. Patients with mild symptoms have a higher risk of developing both common and less frequent complications[2].

Biliary fistula is a rare complication (3%-5%) that is frequently preceded by an episode of acute cholecystitis[3,4]. Biliary fistula is mostly encountered in the duodenum although it can occur anywhere in the gastrointestinal (GI) tract[5]. Finally, just 7%-10% of biliary fistulae cause gallstone ileus, an intestinal obstruction caused by a stone that has migrated through the fistula and stopped anywhere in the GI tract[6]. The terminal ileum and ileocecal valve are the most common locations[7], whereas gastric outlet obstruction from an impacted gallstone named "Bouveret's syndrome", is a very rare complication (1/10 000 cholelithisiasis)[8].

To our knowledge, this is the first case of incomplete gastric outlet obstruction manifested as a "waxing and waning" syndrome for a period of over 20 mo before diagnosis was defined.

## CASE REPORT

A 79-year old woman was referred to our hospital when a recent oesophagogastroduodenoscopy (OGDS) revealed the presence of a foreign body with its maximum diameter > 4 cm, partially obstructing the gastric lumen, firmly attached to the antral greater curvature region where a fistula was partially explorative. The procedure was performed to investigate the patient's two-year old dyspepsia. This mild symptom alternated every two months with bouts of colic pain associated with self-

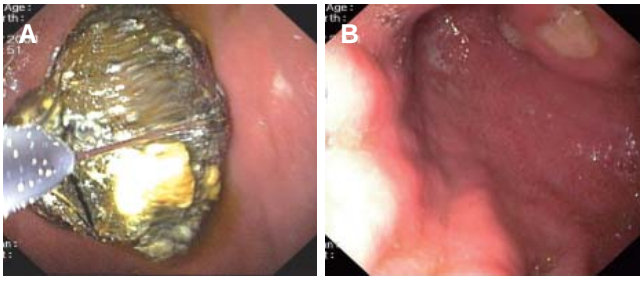


**Figure 1** Oesophagogastroduodenoscopy confirming the presence of a foreign body looking like a biliary stone (**A**) and a 15 mm-wide ulcer (**B**).

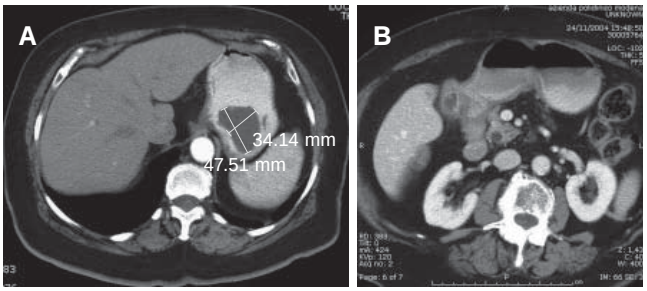


**Figure 2** Computerised tomography revealing the density of foreign body typical of ectopic biliary stone (**A**) and the oedematous wall of the gastric antrum (**B**).

limited vomiting lasting 2 d.

The patient reported an earlier negative colonoscopy, performed due to the migration of gastric foreign body through a gastro-colic fistula. His symptoms occurred after two days of acute abdominal pain associated with vomiting due to acute gastroenteritis. An abdominal ultrasound scan (US) revealed cholelithiasis.

The patient was admitted to our ward in good general clinical conditions. Blood tests were completely normal apart from ferrittin, which was twice the normal range.

An abdominal computerised tomography scan (CT) and another OGDS were then performed. Endoscopic examination confirmed the presence of the foreign body, looking like a biliary stone (Figure 1A). Simple endoscopic lithotomy was not possible as the stone was too large, and an attempt at mechanical fragmentation of the stone was unsuccessful. The foreign body appeared to be firmly attached to the gastric wall, and a 15 mm wide ulcer was visible where the opening of the fistula has been previously described (Figure 1B). The CT revealed that the foreign body had a density typical of an ectopic biliary stone (Figure 2A) and the oedematous wall of the gastric antrum could not be separated from the thickened wall of the gallbladder fundus (Figure 2B).

Further air-fluid level was present in the gallbladder (Balthazar and Schester sign) and the intra- and extrahepatic bile ducts were slightly stretched. Lastly, a magnetic resonance cholangio-pancreatography (MRCP) was performed in the hope of observing the fistula. The procedure was negative (Figure 3). All of these data suggest that a multidisciplinary consultation was needed to decide the most appropriate management for this particular clinical setting.

We eventually opted to attempt "one-stage surgery".

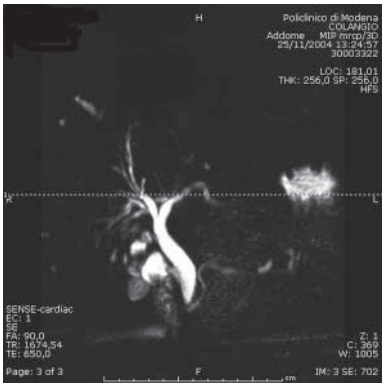


**Figure 3** Magnetic resonance cholangiopancreato-graphy showing no fistula.

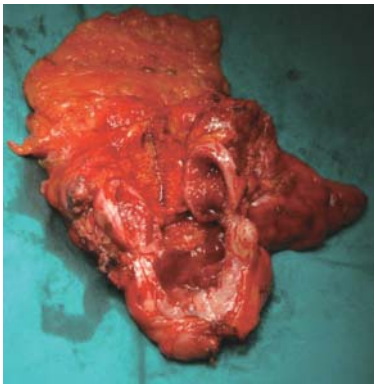

**Figure 4** One-stage surgery with dissection of omental-cholecystic adhesions.

**Figure 5** A large ectopic stone extracted.

After dissection of many omental-cholecystic adhesions, retrograde cholecystectomy was performed successfully (Figure 4). Finally, a gastrostomy was performed on the anterior gastric wall and a large ectopic stone was extracted (Figure 5).

The post-surgical course was regular and during six-month follow-up the patients' general condition was excellent. The patient was completely asymptomatic with normal biliary tree on the US scan.

## DISCUSSION

Like any other gallstone ileus, "Bouveret's syndrome" defined as gastric outlet obstruction caused by a large gallstone occluding the pyloro-duodenal region following a biliogastric or bilioduodenal fistula, primarily affects the geriatric population, with a medium age of 65-75 years and a female predominance (3-16:1)[9-11]. For these reasons, up to 80%-90% of patients have concomitant medical

illnesses (CIC, IRC, diabetes) and the management of such patients should be guided by their general clinical status[4].

The long period of approximately twenty months between the onset and diagnosis of the disease, can be attributed to the patients' carelessness, and the alternating gravity of symptoms combined with the patient's good general health can justify the decision to perform one-stage surgery.

In our case, the particular position of the gallstone led the patient to an insidious clinical setting that urged us to opt for "one-stage surgery" (namely, biliary surgery carried out at the same time as the removal of the intestinal obstruction), which should be still considered as the gold standard for the treatment of gallstone ileus[12]. In fact, it is the only procedure to solve the problem and avoid recurrences and other complications of gallstone disease[13].

In any case, over the past two decades, technological progress has probably made lithotomy or lithotripsy a more conservative approach to reducing patients' risks and has thus been suggested for older patients in poor medical conditions. In our patient, simple endoscopic lithotomy or lithotripsy was not indicated as the stone was excessively large (4 cm) and the inflammatory aspects of the gastric antrum required surgical treatment in order to close the fistula. An attempt at endoscopic mechanical fragmentation of the stone was unsuccessful.

The so-called "two stage surgery" consists of postponing biliary surgery (cholecystectomy and fistula repair) to a later stage with the presence of residual symptomatic stone in asymptomatic subjects[12,24].

Although no patients deny prompt relief from an intestinal obstruction[4], in the case of Bouveret's syndrome, an endoscopic approach represents a reliable alternative to gastro/duodenothomy[15,16]. Preoperative aetiological diagnosis is now possible in most patients and in this case, endoscopic lithotomy and lithotripsy (where possible) represent the first-line approach to treatment as the death rate associated with surgery is still 19%-24%[17,18].

Nevertheless, surgery remains the main procedure in particular situations such as stone impaction in the fistula[19], stone compression of the duodenal wall[20], GI haemorrhage[21] and improper stone manipulation[18,22-25].

In our case, the medical history and endoscopic findings suggested an unusual chronic situation to which the only possible solution was surgery. We, therefore, performed a complete radiological study to better define the anatomy of the lesion before the surgical procedure, including abdominal CT and MRCP of the biliary tree. The first combines the qualities of US and plain X-rays in defining Rigler's diagnostic triad (intestinal obstruction, pneumobilia and ectopic stone)[4,26]. It, therefore, represents the single best imaging technique for the diagnosis of gallstone ileus and Bouveret's syndrome[26]. It may also be helpful in assessing gallbladder wall thickness (signs of acute or chronic cholecystitis), content (air, residual gallstones) and biliary fistula[5,26].

MRCP has recently been proposed as a useful tool for differentiating between fluid and gallstones and also for observing the fistula when sufficient fluid is present[27].

In our case, all the investigations suggested a complicated clinical situation in which an incomplete pyloric occlusion developed following the migration of a large stone through a cholecystogastric fistula, probably due to a poorly-interpreted and treated episode of acute cholecystitis in a case of undiagnosed chronic cholecystitis.

In conclusion, the clinical picture and anatomy of the lesions suggest that a conventional one-stage laparatomic procedure can completely solve the problem, simultaneously avoiding recurrence and other gallstone disease complications.

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*CASE REPORT*

# Case of clear-cell hepatocellular carcinoma that developed in the normal liver of a middle-aged woman

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## Abstract

A 36-year-old woman was admitted to our department for close examination of a liver tumor that was found during a medical checkup. Abdominal US, CT and MRI showed a tumor in segment 7 (S7) of the liver. Although imaging suggested hepatocellular carcinoma, laboratory tests showed no abnormality in liver function, hepatitis virus markers were negative, and tumor markers including protein induced by vitamin K absence or antagonist Ⅱ (PIVKA-Ⅱ), α-fetoprotein (AFP), carbohydrate antigen 19-9 (CA19-9), and carcinoembryonic antigen (CEA) were all within normal ranges. Upon aspiration biopsy of the liver, the histopathological diagnosis was moderately differentiated hepatocellular carcinoma. Therefore, right hepatectomy was performed. Although a part of the tumor was necrotic, about 60% of the viable part showed a clear-cell variant. Consequently, it was diagnosed as clear-cell hepatocellular carcinoma. It was noted that the background liver tissue was normal. This case is worthy of reporting because development of clear-cell hepatocellular carcinoma in the normal liver of a middle-aged woman is rarely seen.



**Key words:** Clear-cell hepatocellular carcinoma; Liver; Middle-aged

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## INTRODUCTION

Hepatocellular carcinoma often develops against a background of chronic viral hepatitis including hepatitis B virus (HBV) and hepatitis C virus (HCV), autoimmune hepatic diseases including autoimmune hepatitis and primary biliary cirrhosis, alcoholic liver injury, and non-alcoholic steatohepatitis (NASH). We report a case of hepatic cancer in which no abnormality existed in liver function, hepatitis virus markers were negative, and the background liver was normal, and which was histopathologically clear-cell carcinoma, in which clear cells accounted for about 60% of the tissue. This patient presented with no background of carcinogenic risk factors, such as viral hepatitis, and showed rare histopathology.

## CASE REPORT

A 36-year-old woman had a medical checkup on October 17, 2005. A tumor was found in S7 of the liver by abdominal US. She was referred and admitted to our department on November 28, 2005 for close examination and treatment. She had a history of alcohol consumption of 1 L beer per day for 15 years. Her family had no history of hepatic disease. On admission, her conjunctivas were not jaundiced, and heart and respiratory sounds were normal. The liver, spleen and tumor were not palpable.

Laboratory tests on admission showed no abnormality such as inflammation or abnormal liver function for peripheral blood and biochemical tests. Hepatitis B surface antigen (HBsAg), hepatitis B core antibody (HBcAb) and HCV antibody (HCVAb) were negative. All tumor markers tested showed normal values: specifically, 16 mAu/mL for PIVKA-Ⅱ (criterion, < 40), 6.2 ng/mL for AFP (criterion, ≤ 10.0), 20.2 U/mL for CA19-9 (criterion, ≤ 37), and 0.9 ng/mL for CEA (criterion, ≤ 5.0). Anti-nuclear and anti-mitochondrial antibodies were negative.

Abdominal US and CT showed a tumor of about 60 mm diameter in S7 of the liver. The lesion was visible on early phase imaging. However, there was also a part that was not visible in the image. It was apparent as a low-density area in the portal phase image (Figure 1A and B). Abdominal MRI showed that the T1-weighted image revealed a well-defined low-intensity area in S7, with a high-intensity part inside. The tumor was depicted as a

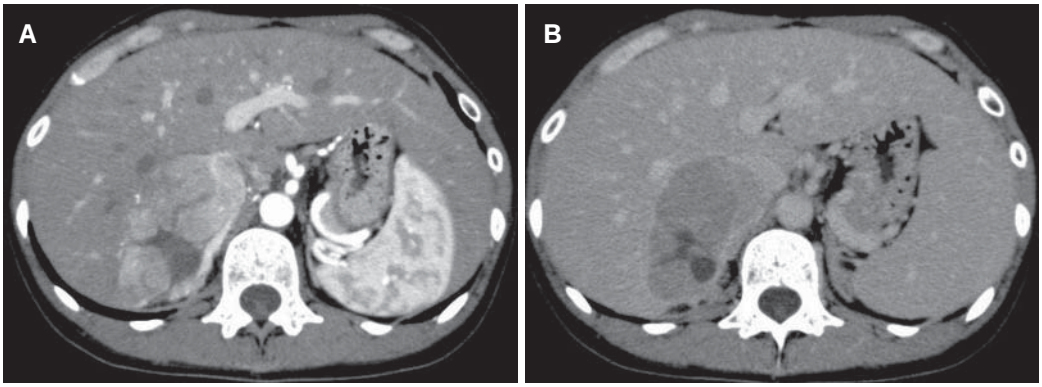


**Figure 1** **A**: Dynamic enhanced CT showed a nodular lesion in the liver that was enhanced in the early image. However, the interior of the mass showed a low-density area; **B**: In the subsequent late phase, the lesion revealed washout of contrast enhancement.

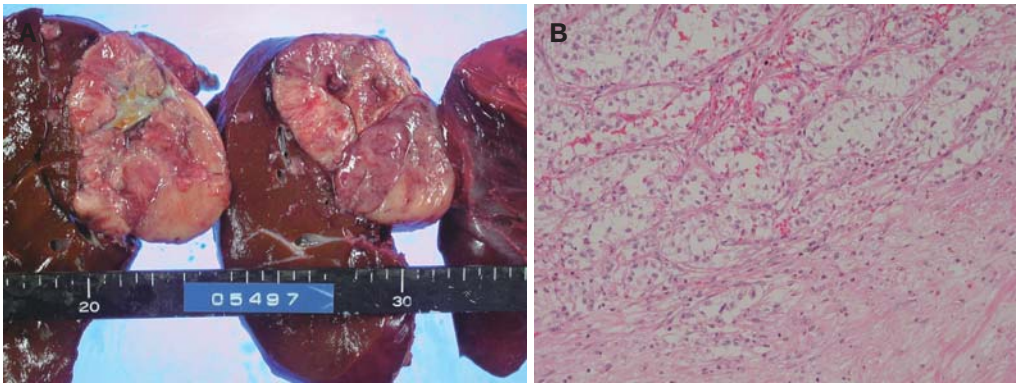


**Figure 2** **A**: The tumor had a fibrous capsule. Its cut surface was whitish, and tumorous tissue had a necrotic part at the center; **B**: Histological examination showed sheet-like growth of clear, atypical cells (HE, × 40).

high-intensity area in the T2-weighted image. Angiography showed that increased tumor vascularity was observed from the right hepatic artery and the inferior phrenic artery to the tumor area. A slight tumor stain was visible in the tumor area in S7 of the liver. Although the imaging results suggested hepatocellular carcinoma, all hepatitis virus and tumor markers were negative, and non-cancerous tissues had no signs of chronic disease.

Consequently, aspiration biopsy of the liver was carried out for definitive diagnosis. Based on histopathology, the patient was diagnosed with moderately differentiated hepatocellular carcinoma, trabecular type, and right hepatectomy was performed on December 27, 2005. A 60-mm tumor was located in the posterior segment of the right hepatic lobe; the tumor was partly adherent to the caudal portion of the adrenal gland. The tumor had a fibrous capsule. Its cut surface was whitish, and tumorous tissue had a necrotic part at the center (Figure 2A). Histopathology showed the border between the tumor and the non-cancerous part formed a fibrous capsule. Approximately 60% of the tumor showed sheet-like growth of clear, atypical cells (Figure 2B). Other areas of the tumor were composed of moderately-differentiated HCC with a trabecular growth pattern. The tumor center was occupied by necrotic tissue.

## DISCUSSION

Hepatocellular carcinoma often develops against a background of chronic liver disease. Specifically, 77.4% and 62.6% of cases in Japan are associated with chronic hepatitis and liver cirrhosis, respectively. Among cases with histopathological diagnosis, only 7.6% have normal liver as the background, as in this present case[1]. Furthermore, HBV- or HCV-antigen-positive liver cirrhosis accounts for about 80% of cases of liver cirrhosis; other causes include primary biliary cirrhosis, autoimmune hepatitis, alcoholic cirrhosis, NASH, and Budd-Chiari syndrome[2]. In the present case, although the patient had a history of alcohol consumption, there was no sign of alcoholic fibrosis in non-cancerous tissues. For that reason, it was presumed pathologically that the hepatocellular carcinoma developed from the normal liver. Liver cancer is considered to be comparatively rare in young persons, and patients < 35 years old account for 0.6% of the cases diagnosed with clinical hepatocellular carcinoma[1]. Moreover, according to data from 1994 to 1995 by the Follow-up Committee, Liver Cancer Study Group of Japan in 1998, 60 cases of liver cancer in young patients aged < 35 years old consisted of 12 HCVAb-positive (20%), 34 HBsAg-positive (57%), three positive cases each (5%) for HCVAb and HBsAg, and 11 virus-marker-negative cases (18%), which indicates that HBsAg is often positive among younger patients with liver cancer[3]. Although virus markers were negative in our case, reports also exist of occult HBV infection, in which serum HBV marker was negative, but HBV existed in the serum and liver tissue[4], and comparatively frequent incorporation of HBV in hepatocellular carcinoma developed in normal liver[5-7]. Therefore, involvement of viruses in this case is not implausible.

Clear-cell hepatocellular carcinoma is not frequent and has been reported to account for 7.5%-12.5% of all liver cancer cases[8,9]. The existence of clear cells, as well as fatty changes, is characteristic of well-differentiated hepatocellular carcinoma in the early stage; its frequency is presumed to

be decreased along with enlargement of the cancer[10]. In a large hepatocellular carcinoma, as that presented in this case, it has been reported that clear-cell hepatocellular carcinoma occurs at a frequency of 0.9%-8.8%[11]. The mechanism of development of clear cells is presumed to involve metabolic disorders and abnormalities of sugar metabolism for reasons including decreased portal blood flow and underdeveloped tumor arteries in the early stage of cancer[10,12]. Histopa-thologically, it is important to distinguish clear-cell hepatocellular carcinoma from liver metastases from other organs, especially renal cell carcinoma, and it can be distinguished by immunostaining[13]. As for the present case, the possibility of malignant tumors derived from other organs was clinically eliminated, and immunostaining of excised samples excluded liver metastasis from renal cancer. On the other hand, fibrolamellar hepatocellular carcinoma and epithelioid hemangioendothelioma are malignant liver tumors that are often seen in young persons without background liver diseases. However, the present case did not have the same histopathology as those diseases. Prognosis of clear-cell hepatocellular carcinoma has been reported to be better than[8,14], the same as, or worse than[9,12] that of common hepatocellular carcinoma. Further careful follow-up observations are needed in the future.

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*CASE REPORT*

# Anaplastic carcinoma associated with a mucinous cystic neoplasm of the pancreas during pregnancy: Report of a case and a review of the literature

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## Abstract

Oncogenesis of anaplastic carcinoma of the pancreas is a subject of controversy, because it shows sarcomatous nature with extremely poor prognosis. We herein report an unusual case of anaplastic carcinoma occurring with a recurrent mucinous cystic neoplasm in a 38-year-old female. A 10-cm retroperitoneal cystic mass was pointed out in the first pregnancy and a probable diagnosis of mucinous cystic neoplasm was made in October 2000. She refused surgery first and delivered her baby uneventfully. During her second pregnancy in 2002, however, she presented hematemesis and underwent urgent distal pancreatectomy, splenectomy and partial resection of the gastric wall where the tumor perforated. A diagnosis of borderline-type mucinous cystic neoplasm with ovarian-like stroma was made. Nine months later, CT visualized a recurrent cystic tumor near the pancreatic stump, which was subsequently resected. Pathology revealed that the tumor was composed of two different components of borderline-type mucinous cystic neoplasm and anaplastic carcinoma. The latter was intensely positive for vimentin, CD68, p53 and focally for cytokeratin, suggesting both sarcomatous and carcinomatous differentiation. She survived four years after the second surgery without tumor recurrence. Although the origin of anaplastic carcinoma has not been determined yet, it should be remembered that anaplastic carcinoma can occur in association with mucinous cystic neoplasm of more benign histology.



**Key words:** Mucinous cystic neoplasm; Anaplastic carcinoma; Pancreatic tumor; Ovarian-like stroma; Pregnancy

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## INTRODUCTION

Anaplastic carcinomas of the pancreas are rare, representing only 0.5%-7% of all non-endocrine malignances in this organ[1,2]. The origin of the tumor is considered pancreatic ductal cells because they are accompanied with areas of adenocarcinomas in most cases[2]. However, they also contain mesenchymal components such as spindle-shaped cells and osteoclastoid giant cells, reminiscent of having sarcomatous differentiation. The prognosis of pancreatic anaplastic carcinoma is extremely poor. The combination of immature adenocarcinoma and sarcoma components may portend an adverse clinical course.

Mucinous cystic neoplasms of the pancreas, on the contrary, have an indolent clinical course and a much better prognosis than anaplastic carcinoma if respectable[3]. Mucinous cystic neoplasms occur predominantly in women and often have an ovarian-like stroma that contains spindle cells commonly observed in the ovary. The association of anaplastic carcinoma with mucinous cystic neoplasm has rarely been reported to date.

We report an unusual case of pancreatic anaplastic carcinoma coexistent with recurrent mucinous cystic neoplasm in a 38-year-old female, who previously underwent distal pancreatectomy for a borderline-type mucinous cystic neoplasm during her second pregnancy.

## CASE REPORT

A 38-year-old female patient with no previous medical history was introduced to our hospital in October 2000, for further examination of a retroperitoneal cystic tumor that was disclosed incidentally by routine ultrasonography (US) in the first trimester of her first pregnancy. She was completely asymptomatic. Magnetic resonance imaging (MRI) and US showed a large multiloculated cystic tumor with a thick capsule at the tail of the pancreas. It measured 10 cm in diameter and displaced the stomach

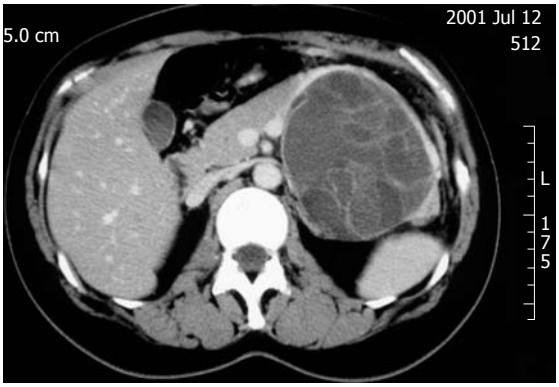


**Figure 1** CT after the first delivery showing a 10 cm × 10 cm multi-loculated cystic tumor at the tail of the pancreas.

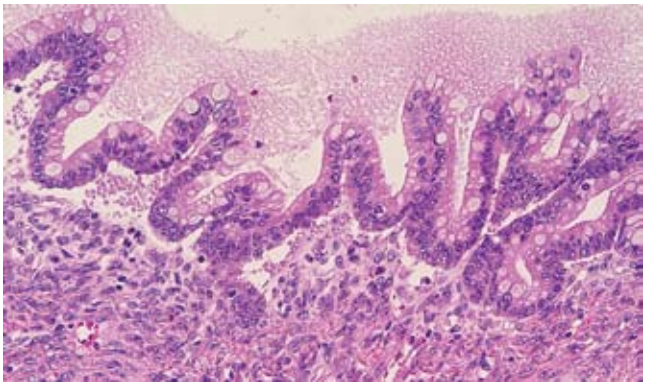

**Figure 2** Consistent pathology of the primary tumor with a borderline-type mucinous cystic neoplasm (HE staining, original magnification, × 100).

anteriorly. A probable diagnosis of a mucinous cystic neoplasm was made. We recommended her to receive resection of this potentially malignant tumor soon, but she decided to postpone operation until delivery. She delivered a healthy baby in July 2001. Computed tomography (CT) showed a multiloculated cystic tumor (Figure 1), which seemed similar in size of that at the initial diagnosis. She hesitated to receive surgery and was lost to medical follow-up.

In September 2002, she presented repetitive vomiting and hematemesis, when she noticed her second pregnancy. In November, She presented hematemesis and tarry stool again. MRI and US showed marked enlargement of the cystic tumor. Because of progressive anemia, she underwent urgent operation in the second trimester. At laparotomy, a dense adhesion between the tumor and posterior wall of the stomach was noted, so that an en-block resection was accomplished by distal pancreatectomy, splenectomy and a partial resection of the stomach. The postoperative course was uneventful and she delivered her second baby in April 2003.

In August 2003, a routine medical checkup by US disclosed a 3 cm tumor near the pancreatic stump. The tumor showed both cystic and solid appearances with capsule enhancement. The second operation was performed under the diagnosis of local recurrence of the mucinous cystic neoplasm. At laparotomy, the tumor was located between the pancreatic stump, posterior wall of the stomach and the left adrenal, which was removed en-block by additional partial pancreatectomy, partial gastrectomy, and left adrenalectomy.

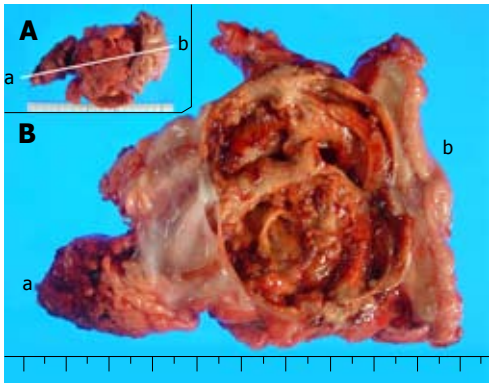


**Figure 3** Cut surface of the recurrent tumor. **A:** Location of the cystic tumor between the pancreatic stump (a) and posterior wall of the stomach (b); **B:** Cut surface of the specimen revealing the tumor composed of two different components.

The patient was still alive and well four years after the resection. CT revealed no local and distant tumor recurrences at the latest follow-up.

### *Pathological findings*

The tail of the pancreas resected at the first operation contained a multiloculated cystic tumor, measuring 14 cm × 14 cm, filled with about one liter of straw-yellow mucinous fluid. The cyst was uniformly lined with smooth inner surface with a thickened capsule of 5-10 mm width. At the cut surface of the maximal dimension, ten blocks were sampled for microscopic examination. The histological diagnosis was a mucinous cystic neoplasm, borderline type (Figure 2). The tumor had an ovarian-like stroma which was immunohistochemically positive for a progesterone receptor. None of the lymph nodes was involved, nor was the pancreatic cut margin. At the site of perforation to the stomach, no neoplastic change was identified in the gastric wall.

The recurrent tumor was entirely cystic and contained brown, dirty mucus. The tumor was located between the pancreatic stump and posterior wall of the stomach. Macroscopically, it was composed of two different parts. At the pancreatic side, the inner surface was white and smooth, resembling that of the previously resected primary tumor, while at the gastric side, it showed a tan-brown, irregular one. There was no transition area between the two parts (Figure 3). Pathology of the part near the pancreatic stump was a borderline-type mucinous cystic neoplasm. The part at the gastric side, on the other hand, contained an anaplastic carcinoma with osteoclastoid giant cells, atypical spindle-shaped cells and round cells, showing sarcomatous changes (Figure 4). The immunohistochemical the features of anaplastic carcinoma are summarized in Table 1. The recurrent tumor was also positive for progesterone receptor. All components of the anaplastic carcinoma were intensely positive for vimentin. Leukocyte common antigen and CD68 were positive in spindle-shaped cells and giant cells, while cytokeratin was positive in cytoplasm of the obviously epithelial component and

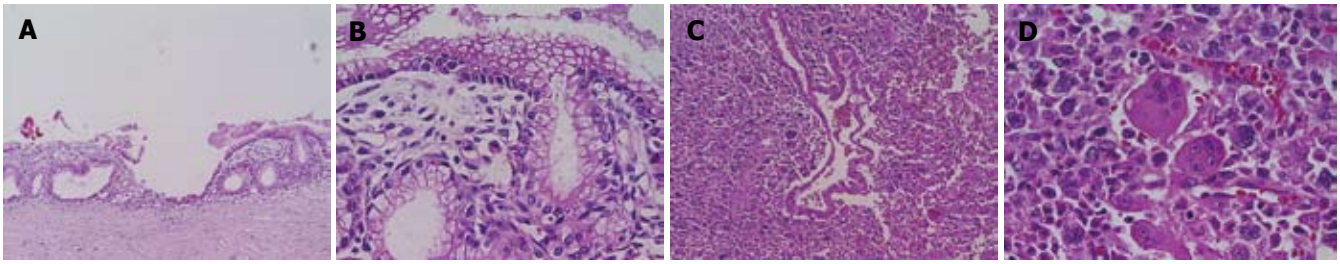


**Figure 4** Pathology of the recurrent tumor at the pancreatic side (as pointed by a in Figure 3) revealing a borderline-type mucinous cystic neoplasm (HE, **A**: x 100, **B**: × 400) and at the gastric side (as pointed by b in Figure 3) an anaplastic carcinoma with giant cells, atypical spindle cells and round cells (**C**: × 100, **D**: × 400).

**Table 1 Immunohistochemical features of each component in anaplastic carcinoma**

| | Adenocarcinoma | Spindle cell | Giant cell |
|---|---|---|---|
| Vimentin | + | + | + |
| Leukocyte common antigen | - | + focal | + focal |
| CD68 | - | + | + |
| Cytokeratin | + | + focal | - |
| P53 | + | + | + |

focally in some of the spindle-shaped cells. P53 was also positive throughout of anaplastic carcinoma.

## DISCUSSION

Anaplastic carcinomas associated with mucinous cystic neoplasms are very uncommon. Only several cases have been reported to date (Table 2)[3-12]. Some underwent

**Table 2 Reported cases of anaplastic carcinoma occuring in association with MCNs**

| No | Author (reference) | Case (age/sex) | Diagnosis | Anaplastic carcinoma | | | | | | | Outcome (survival time) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Tumor location | Size (cm) | Spindle cell | Giant cell | Adeno-carcinoma | Invasion | lymphatic involvement | Distant metastasis | |
| 1 | Compagno J[3] | 32/F | Anaplastic carcinoma derived from MCN | Body | ND | ND | ND | + | ND | ND | - | Dead (3 yr) |
| 2 | Gracia Rego JA[4] | 45/F | Mucinous cystadenocarcinoma with pseudosarcomatous mural nodules | Body-tail | 11 | + | ND | + | Capsule | ND | - | Dead (16 mo) |
| 3 | Hartz PH[5] | 56/F | Cystadenocarcinoma with anaplasia | Tail | 6 | + | + | ND | Capsule, peripan-creatic nerve | ND | Liver, peri-toneum | Dead (autopsy) |
| 4 | Lane RB[6] | 25/F | Anaplastic carcinoma with MCN | Tail, liver, lymph node | 15 | + | - | + | ND | + | Liver | ND |
| 5 | Logan SE[7] | 23/F | Pleomorhic adenocarcinoma originated from MCN | Body-tail | 17 | + | ND | + | Stomach | + | Liver | Dead (1 mo) |
| 6 | Marinho A[8] | 70/F | Mucinous cystadenocarcinoma with a mural nodule of anaplastic carcinoma | Body-tail | 4.5 | ND | ND | + | Capsule | ND | - | ND |
| 7 | Sommers SC[9] | 48/M | Cystadenocarcinoma with foci of pleomorphic carcinoma | ND | ND | ND | ND | + | + | ND | + | - | Dead (6 mo) |
| 8 | Tsujimura T[11] | 43/F | Malignant histiocytoma with mucinous cystadenoma | Tail | 16 | + | + | ND | ND | ND | - | ND |
| 9 | [1]Wenig BM[12] | 67/M | MCN with sarcoma stroma | Tail | 19 | + | ND | + | ND | ND | ND | Dead (15 mo) |
| 10 | [1]Wenig BM[12] | 48/F | MCN with sarcoma stroma | Tail | ND | + | ND | + | ND | ND | ND | Alive (12 mo) |
| 11 | [1]Wenig BM[12] | 65/F | MCN with sarcoma stroma | Tail, peritoneum | 30 | + | ND | + | Vascular | ND | Omentum, pleura | Dead (9 mo) |
| 12 | Hakamada K | 39/F | Anaplastic carcinoma with recurrent MCN, borderline-type | Tail | 5 | + | + | + | - | - | - | Alive (4 yr) |

ND: Not described; MCN: Mucinous cystic neoplasm. [1]Later re-analyzed genetically and reported by van den Berg W[11].

resection of the cystic tumor at the onset, while others were treated with cystenterostomy first. Because of co-existent adenocarcinomas in the areas of anaplastic cancer, most of the tumors were thought to be of epithelial origin. However, the pathogenesis of these neoplasms, particularly those with epithelial and mesenchymal components, remains a subject of controversy.

In our case, the entire anaplastic carcinoma was intensely immunohistochemical positive for vimentin, an intermediate fragment associated with mesenchymal cells. Areas of spindle-shaped cells and giant cells were also positive for leukocyte common antigen and CD60. These facts suggest the sarcomatous nature of the anaplastic carcinoma[12]. In addition to the positive vimentin, areas of the carcinoma were positive for cytokeratin. The co-expression of cytokeratin and vimentin in the cells with the same hisotological character seemed unusual, but it was reported that vimentin becomes positive when the tumor cells are in a proliferative state[13,14]. Immunohistochemical study alone cannot determine the origin of anaplastic carcinoma in the mucinous cystic neoplasms. Van den Berg *et al*[11] analyzed genetically three cases of anaplastic carcinoma related to mucinous cystic neoplasms, and disclosed that the sarcomatous and carcinomatous components are different phenotypically but genetically uniclone.

It was reported that malignant fibrous histiocytoma coexists with mucinous cystic adenoma[10], suggesting that sarcomas can occur from a relatively benign epithelial histology of mucinous cystic neoplasms. Indeed, the origin of sarcomas and carcinomas in mucinous cystic neoplasm is still unknown, but the theory that both sarcomas and carcinomas are originated from primitive mesenchymal stem cells with totipotential properties in the mucinous cystic neoplasm, might be another acceptable hypothesis for the histogenesis of anaplastic carcinoma.

Another interesting feature of this case is that the tumor experienced pregnancy twice. To the best of our knowledge, there are no reports on the relation of the progression of mucinous cystic neoplasm with pregnancy. Ovarian-like stroma exists exclusively in female patients with mucinous cystic neoplasms, which often are immunohistologically positive for progesterone and/or estrogen receptors. In our case, both the primary and recurrent tumors were positive for progesterone receptors. It is unknown whether physiological changes in blood concentration of these hormones promote the progression of the tumor. In our patient, the tumor size was stable during the first pregnancy, but rapidly enlarged in the second trimeseter of the second pregnancy. Increased mucious production might induce the enlargement of the tumor and subsequent rupture.

Our patient was still alive without any sign of recurrence four years after the second operation. However, close monitoring on tumor recurrence should be required because anaplastic carcinoma reportedly has a dismal prognosis.

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*CASE REPORT*

# Exophytic inflammatory myofibroblastic tumor of the stomach in an adult woman: A rare cause of hemoperitoneum

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## Abstract

Inflammatory myofibroblastic tumor (IMT) of the stomach in adults is extremely rare, with unpredictable prognosis. We present a 55-year-old woman with a gastric IMT. She experienced sudden abdominal pain 4 d previously. Physical examination showed mild abdominal tenderness in the hypogastrium, but no palpable abnormal abdominal mass. Abdominal CT showed a mass of approximately 8 cm in the gastrocolic ligament. On laparoscopic exploration, unexpected hemoperitoneum of approximately 1.5 L of blood was found, and an exophytic gastric mass of approximately 10 cm, appeared from the anterior wall of the gastric body along the greater curvature. Laparoscopy further showed that non-clotting blood in the abdominal cavity seemed to be from the gastric tumor. After conversion to open surgery for more precise evaluation of the cause of hemoperitoneum and the large friable tumor, gastric wedge resection, including the tumor, was conducted. The final diagnosis was consistent with IMT that originated from the gastric wall.



**Key words:** Stomach disease; Stomach neoplasms; Hemoperitoneum; Myofibroma; Granuloma; Plasma cell; Stomach surgery

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## INTRODUCTION

Inflammatory myofibroblastic tumor (IMT) is a rare neoplasm. The perspective of this distinctive disease has changed over time from a reactive, inflammatory process to a neoplasm of intermediate biologic potential[1,2]. Even though IMT has recently been known to span the entire age range and can arise from any site in the body[1], gastric IMT in an adult is, nevertheless, still very rare. We present a case of gastric IMT in an adult and a review of the literature.

## CASE REPORT

A 55-year-old woman presented with acute-onset, sharp abdominal pain. The pain had developed 4 d prior to referral to our department from a local hospital for further evaluation of abdominal pain. On admission, her vital signs were normal. She denied other gastrointestinal symptoms and signs such as nausea, vomiting, abnormal bowel habits, melena or hematochezia, except for sharp abdominal pain. She also denied weight loss, fever, or other systemic symptoms. She had no medical or family history. Physical examination showed mild abdominal tenderness in the hypogastrium, but no palpable abnormal abdominal mass. Her laboratory findings, including tumor markers, were unremarkable, except for a normocytic, normochromic anemia: hemoglobin, 9.86 g/dL; hematocrit, 28.6%; mean corpuscular volume, 100.4 fL; and elevated erythrocyte sedimentation rate, 54 mm/h. The chest and abdominal X-ray films revealed no abnormal findings. Abdominal CT demonstrated a mass of approximately 8 cm in the gastrocolic ligament or gastric wall (Figure 1), and abdominal MRI showed a heterogeneous mass of approximately 8 cm in the gastrocolic ligament or gastric wall (Figure 2).

Endoscopic examination, including colonoscopy, showed no luminal or mucosal lesion and no remarkable

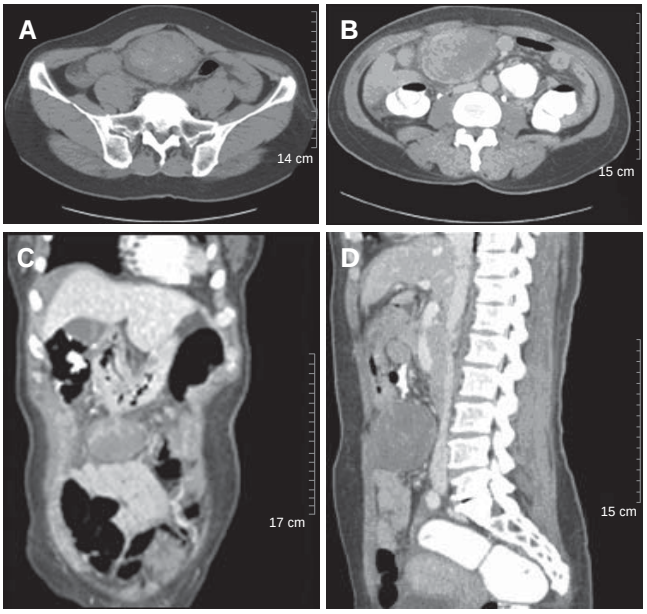


**Figure 1** Abdominal CT revealed a large solid mass at the gastrocolic ligament or the gastric wall, which showed heterogeneous density on an non-enhanced image (**A**). The 8 cm mass showed internally enhanced vessels on the arterial phase of CT and delayed peripheral enhancement of the mass on the venous phase (**B**-**D**).

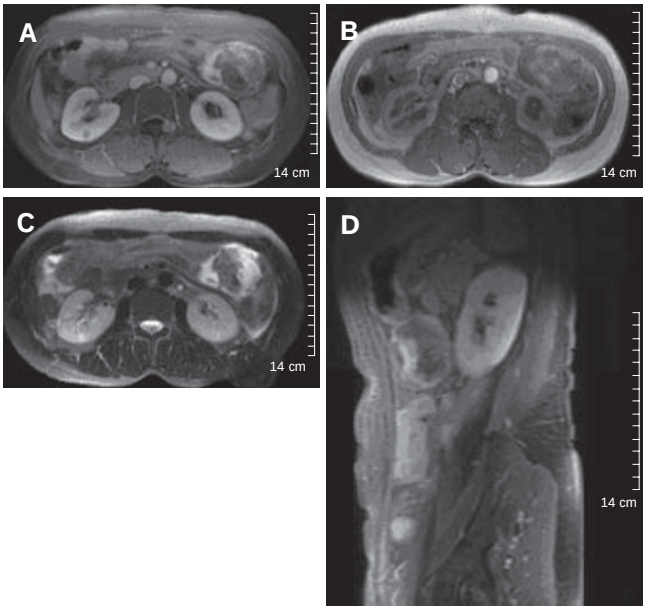


**Figure 2** Contrast-enhanced MRI of the abdomen showed a mass of approximately 8 cm, seen at the left upper quadrant of the abdomen. The margin of the mass was lobulating, and it was attached to the greater curvature of the stomach. It contained a peripheral enhanced solid portion and a central non-enhancing portion (**A**). Signal intensity of the central non-enhancing portion was low on T1WI (**B**) and T2WI (**C** and **D**), which suggested internal hemorrhage within the tumor.

findings, except chronic atrophic gastritis with *Helicobacter* infection. Routine gynecological evaluation was unremarkable. After the work up for the intra-abdominal mass, her abdominal pain completely subsided at the time of operation.

The patient underwent laparoscopic exploration. Unexpected hemoperitoneum of approximately 1.5 L of non-clotting blood was found. An exophytic gastric mass from the anterior wall of the gastric body along the greater curvature appeared, similar in appearance to the spleen or a hematoma, approximately 10 cm in size (Figure 3). Laparoscopic diagnosis was massive intra-abdominal hemorrhage from the gastric tumor or ectopic spleen. Since further evaluation of the precise cause of hemoperitoneum was needed, and because it would have been difficult to remove the large friable tumor from the abdominal cavity, even after a safe laparoscopic resection we decided to convert to open surgery.

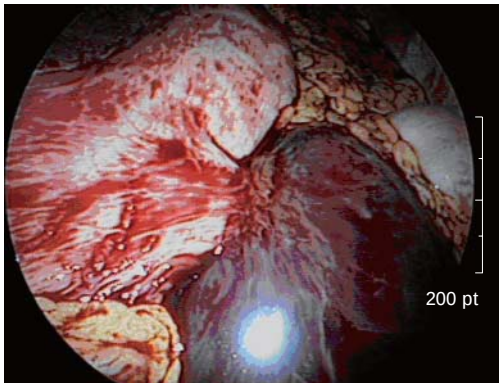


**Figure 3** Laparoscopic view of the exophytic gastric mass with a large amount of intra-abdominal non-clotting hemorrhage.

After evacuation of blood, the abdominal cavity was thoroughly explored for any other source of bleeding, but none was found; the permeated or ruptured exophytic gastric mass was the cause of hemoperitoneum, because preoperative imaging studies, including gynecologic evaluation during admission, showed no evidence of intra-abdominal fluid, and also no other identified focus of bleeding upon surgical exploration. The tumor showed a dark reddish pedunculated mass with a stalk that originated from the gastric wall, but there was no blood vessel directly into the tumor. During dissection, we could not find any tear or rupture on the exophytic tumor. Gastric wedge resection, including the tumor and greater omentum, was conducted.

Grossly, the external surface of a well-encapsulated lump of soft solid tumor was smooth and glistening, but there was no gastric mucosal lesion (Figure 4A). The tumor measured 8.5 cm × 7.1 cm × 3.6 cm and weighed 88.1 g, and its stalk measured 2.0 cm × 1.2 cm × 1.9 cm. On serial sectioning, the cut surface was characterized by several amorphous fragments of parenchymal tissue, which were separated by the cystic spaces (Figure 4B). Histologically, the tumor was composed of round and spindle-shaped myofibroblastic cells, diffusely scattered inflammatory cells, and many vascular structures (Figure 5A). The mitotic count was 1/10 high power fields (HPF). The tumor cells showed positive immunoreactivity for vimentin (Figure 5B), while being negative for c-kit, CD34, desmin, smooth muscle actin (SMA), S-100, anaplastic lymphoma kinase (ALK), β-catenin, and CD31. Ki-67 labeling index was approximately 10%. Lymph nodes found along the gastroepiploic vessels in the omentum were all negative for tumor. The final pathologic diagnosis was consistent with IMT that originated from the gastric wall.

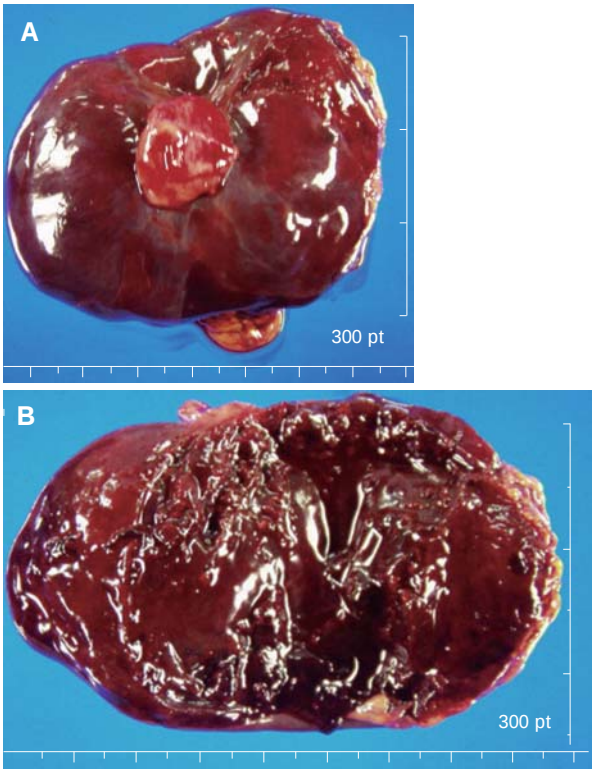


**Figure 4** The external surface of a well-encapsulated lump of soft solid tumor, weighing 88.1 g, was smooth and glistening, but showed no gastric mucosal lesion (**A**). Cross-sectional surface of the tumor was characterized by several amorphous fragments of parenchymal tissue, which were separated by the cystic spaces (**B**).

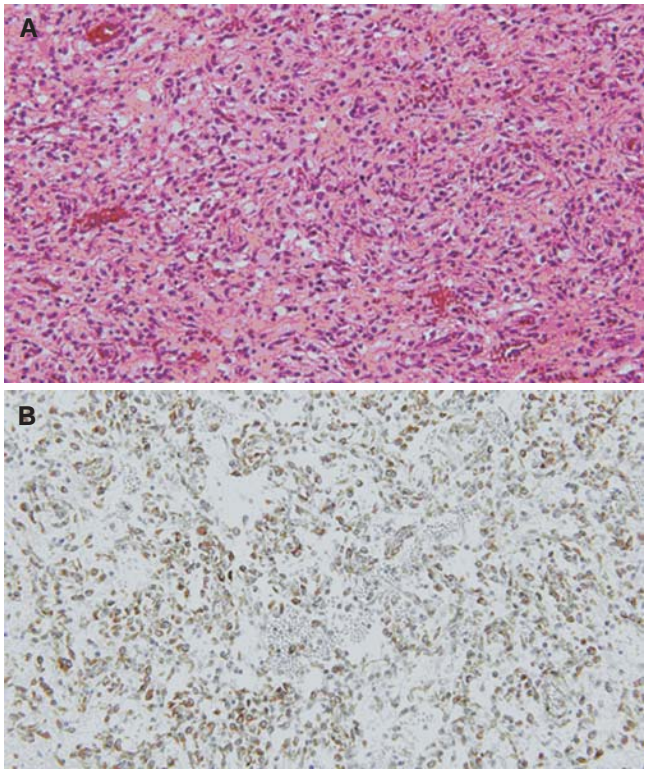


**Figure 5** **A**: The tumor was composed of round and spindle-shaped myofibroblastic cells. Diffusely scattered inflammatory cells and many vascular structures are seen (HE, × 400); **B**: The tumor cells showed positive immunoreactivity for vimentin (× 400).

The patient had an uneventful postoperative course and has been followed up, including positron emission tomography, for any recurrence.

## DISCUSSION

IMT is a rare, distinctive disease. Various terms such as inflammatory pseudotumor, plasma cell granuloma, inflammatory myofibroblastoma, and inflammatory myofibrohistioblastic proliferation have previously been used to describe the disease[3], which indicates that the exact nature of IMT is not yet fully understood. It has been debated whether IMT is a tumor or inflammation, and also whether it is benign or malignant[1]. However, recent studies on cytogenetic abnormalities, such as rearrangements of the ALK gene on chromosome 2p23[4,5], clonal chromosome abnormalities[6-8], and DNA aneuploidy[9], and the role of oncogenic viruses[10,11] in the pathogenesis of IMT suggest that it is a true neoplasm. According to the current classification of the World Health Organization[12], IMT is a neoplasm with a tendency for local recurrence and a very low rate of metastasis, and is histopathologically composed of myofibroblastic spindle cells, with inflammatory cell infiltrate of plasma cells, lymphocytes and eosinophils.

It was once accepted that IMT is primarily a disease of children and young adults and commonly occurs in the lungs[13,14]. However, a recent study by Coffin *et al* has shown that IMT may span the entire age range and can occur in any site of the body[1]. Nevertheless, gastric IMT in adults is still a very rare disease. Only four case reports of gastric IMT in adults exist in the English literature: Kim *et al* have reported a gastric IMT with peritoneal dissemination in a young adult[15]; Al-Taie *et al* have reported a rapidly growing inflammatory tumor after triple therapy for benign gastric ulcer[16]; Leon *et al* have experienced an IMT of the gastric remnant in a 50-year-old woman with a prior gastrectomy[3]; and Kojimahara *et al* have described a large, poorly demarcated, elevated IMT with infiltrative proliferation of spindle cells over the full thickness of the gastric wall in a 19-year-old woman[17].

This is believed to be the first case report of an exophytic gastric IMT that spontaneously bled into the peritoneal cavity and developed into hemoperitoneum. The present case can be compared with other intra-abdominal IMTs that present with an abdominal mass and related compressive symptoms, such as abdominal pain and vomiting[3,15-18].

Most IMTs require surgery to obtain definite diagnosis and cure. Complete resection is the preferred option, because incomplete excision has been shown to be a risk factor for recurrence[13]. As evident in this case, microrupture of the solid tumor might be another risk factor for early recurrence.

The main difficulty in the management of IMT lies in the unpredictable postoperative course. There are no definitive clinical, histopathological, or genetic features to predict recurrence or metastasis. Recently, reactivity of ALK has been reported to be a favorable prognostic indicator[1]. Differentiation between aggressive and non-

aggressive forms of IMT remains to be further clarified.

We reported a case of gastric IMT that spontaneously bled into the peritoneal cavity during admission. The patient is currently undergoing careful follow-up, because it is not clear whether gastric IMT is benign.

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*CASE REPORT*

# Hyperinsulinemic hypoglycemia due to diffuse nesidioblastosis in adults: A case report

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## Abstract

Persistent hyperinsulinemic hypoglycemia is caused most commonly by an insulinoma in adults or by nesidioblastosis in neonates. In adults, nesidioblastosis is a rare disorder characterized by diffuse or disseminated proliferation of islet cells. We recently encountered a case of nesidioblastosis in an adult. A 71-year-old man was admitted due to intermittent general weakness, abdominal pain, and mild dyspnea. The patient underwent a subtotal gastrectomy for a gastric adenocarcinoma two years ago. After 5 d of admission, the patient showed symptoms of cold sweating, chilling, and hypotension 30 min after eating. Thereafter, he frequently showed similar symptoms accounting for hypoglycemia regardless of food consumption. Laboratory findings revealed a low fasting blood glucose level (25 mg/dL), and a high insulin level (47 μIU/mL). Selective intra-arterial calcium stimulation with hepatic venous sampling (ASVS) was performed to localize a mass and revealed an increased insulin level about four-fold that of the normal fasting level at 60 s in the splenic artery, which suggested the presence of an insulinoma in the tail of pancreas. A distal pancreatectomy was performed. Neither intraoperative exploration nor a frozen biopsy specimen detected any mass-forming lesion. On the histological examination, many of the islets were enlarged and irregularly shaped in all specimens, the arrangement of which was a lobulated islet pattern. Cytologically, a considerable subpopulation of endocrine cells showed enlarged and hyperchromatic nuclei. By immunohistochemistry, the cells were identified as β-cells. These clinical, radiological, microscopic and immuno-histochemical findings are consistent with diffuse nesidioblastosis in adults.



**Key words:** Hyperinsulinemic hypoglycemia; Nesidioblastosis; Adult

## INTRODUCTION

Nesidioblastosis is a term originally conceived by Laidlaw[1] who described the neoformation of the islets of Langerhans from the pancreatic ductal epithelium. This disease is a rare disorder of infants characterized by persistent hypoglycemia as a result of hypersecretion of insulin from β-cell hyperplasia of the pancreas[2]. First described in neonates, it is widely recognized as the primary cause of persistent hyperinsulinemic hypoglycemia in infants[3]. However, in adults, hyperinsulinemic hypoglycemia is caused mostly by an insulinoma, and onset nesidioblastosis in adults represents 0.5%-5% of cases of organic hyperinsulinemia[4,5]. Since the first reported series of onset nesidioblastosis in adults by Harness *et al* in 1981[6], limited cases have been reported to date[7-9].

We report herein a very rare case of hyperinsulinemic hypoglycemia of an elderly man, which was negative in a localizing test for mass and positive in a selective arterial calcium infusion (SACI) test. He was found to have nesidioblastosis during a partial pancreatectomy.

## CASE REPORT

A 71-year-old man was admitted to our hospital due to intermittent general weakness, abdominal pain, and mild dyspnea. The patient underwent a subtotal gastrectomy for a gastric adenocarcinoma 2 years ago. He had no history of diabetes mellitus or hypoglycemia. The patient had discomfort of the abdomen accompanying general weakness several months ago. After 5 d of admission, the patient showed abrupt symptoms of cold sweating, chilling, and hypotension 30 min after eating. These symptoms were relieved after intravascular administration of 50% glucose. Thereafter, the patient frequently showed similar symptoms accounting for hypoglycemia regardless of food consumption.

Laboratory findings determined when the symptoms were present revealed a low fasting blood glucose level (25-48 mg/dL), and a high insulin level (38-47 μIU/mL). A

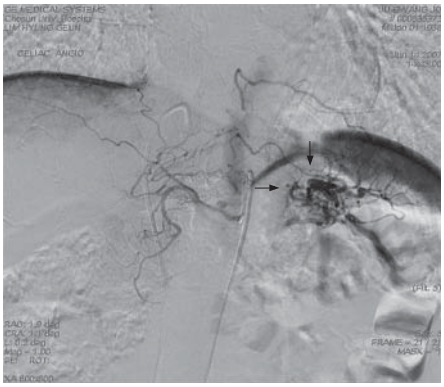

**Figure 1** Celiac angiography showing a hypervascular mass-like lesion in the pancreatic tail area (arrows).

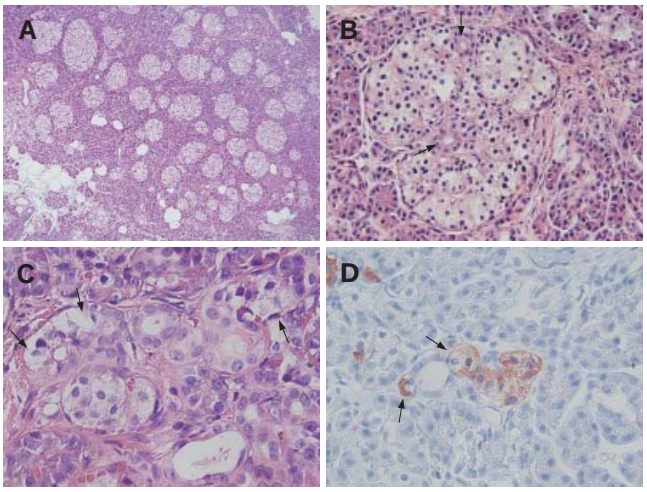


**Figure 2** Irregularly sized-dysplastic islets scattering randomly throughout the pancreas (**A**), islets in intimate association with ducts forming a so-called ductulo-insular complex (arrows indicate ductules within the islet) (**B**), islet cells (arrows) budding off the duct epithelium (**C**), insulin-positive islet cells (arrows) budding off the duct epithelium (immunohistochemical stain for insulin) (**D**).

72-h fasting glucose study failed because of the occurrence of hypoglycemic shock 4 h after commencement of the test.

Endocrine examinations to exclude other causes of hypoglycemia, such as hypopituitarism and adrenal insufficiency, were within the normal range. The above-described symptoms and the results of serological tests were consistent with those of insulin-producing lesions including an insulinoma. However, imaging studies including computed tomography and a sonogram of the abdomen failed to detect a mass except for a highly vascularized area in pancreatic tail (Figure 1). To exclude any possible occult insulinoma, selective intra-arterial calcium stimulation with hepatic venous sampling (ASVS) was performed. After calcium gluconate (0.05 mg/kg body weight) was injected into the splenic, hepatic, gastroduodenal, and superior mesenteric arteries, blood samples were collected from the right hepatic vein every 30 s for 120 s. A selective arterial calcium injection test (SACI) to the left splenic artery increased the insulin level of about 4-fold over the pre-stimulated level. In contrast to the left splenic artery, no significant increment was induced by the SACI to the hepatic, gastroduodenal, and

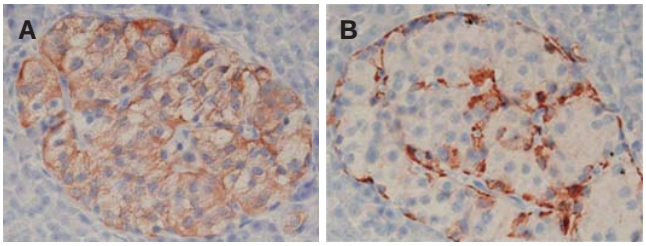


**Figure 3** An immunohistochemical study showing increased insulin producing β-cells (**A**), glucagon producing α-cells (**B**).

superior mesenteric arteries, suggesting the presence of an insulinoma in the tail of pancreas. Under the assumptive diagnosis of an insulinoma of pancreatic tail based on the ASVS test, a distal pancreatectomy was performed. Neither intraoperative exploration nor a frozen biopsy specimen detected any mass-forming lesion. Grossly, the resected pancreas appeared normal, and there was no mass lesion in serial section specimens.

In a histopathological study, a number of dysplastic islets were randomly scattered throughout the pancreatic parenchyma, and their contour and size were markedly variable as compared to the normal pancreatic parenchyma. Ductuloinsular complexes and insulin-positive cells budding off the duct epithelium were also observed (Figure 2). Focally, the distribution of islets was densely crowded. In the majority of islets, multiple β-cells with enlarged and hyperchromatic nuclei and abundant clear cytoplasm were identified (Figures 2 and 3). Immunohistochemically, the number of insulin-secreting β-cells was increased, and the number of glucagon-secreting α-cells was decreased (Figure 3). These clinical, radiological, histopathological, and immunohistochemical findings were consistent with those of nesidioblastosis.

In the post-operative course, the glucose and insulin levels in the patient were well controlled and uneventful for two weeks after surgery. However, beyond that time, the patient repeatedly showed hyperinsulinemic hypoglycemia with no response to medication.

## DISCUSSION

Nesidioblastosis is the name given to the presence of islets in intimate association with ducts, formation of so-called ductulo-insular complexes[10,11]. In adults, an insulinoma accounts for most cases of hyperinsulinemic hypoglycemia[4,12]. Nesidioblastosis has mainly been described in neonates[3]. Since Harness *et al*[6] first described nesidioblastosis in adults, it has only been reported in association with other diseases, such as Zollinger-Ellison syndrome, multiple endocrine adenomatosis, β-cell adenomatosis, Lindau's disease, cystic fibrosis, insulinomas, pancreatic transplantation, orbital lymphoma with hypopituitarism and adrenal insufficiency, familial adenomatous polyposis, hypergastrinemia, and pancreatic polypeptidemia[7].

The morphological criteria[13,14] for establishing its diagnosis are the presence of differently-sized islets often with somewhat irregular outline, and irregularly sized and poorly defined endocrine cell clusters scattered in the acinar parenchyma and often intimately connected with

small or large ducts (ductuloinsular complexes). Another feature is a distinct islet cell hypertrophy with nuclear enlargement, often resulting in the presence of giant and bizarre nuclei. As seen by immunohistochemistry and electron microscopy, these cells are found to be insulin-producing cells. The histopathological findings in the present case were compatible with these criteria. Nesidioblastosis is classified into focal and diffuse types characterized by different clinical outcomes[15]. Focal nesidioblastosis exhibits nodular hyperplasia of islet-like cell clusters, including ductuloinsular complexes and hypertrophied insulin cells with giant nuclei. In contrast, diffuse nesidioblastosis involves the entire pancreas with irregularly sized islets[10].

Sporadic hyperinsulinemic hypoglycemia is the main clinical feature of nesidioblastosis. The present patient had frequently hypoglycemic episodes mostly in the afternoon and night, independent of food consumption. It should be noted that a gastrectomy may evoke hypoglycemia, which is sometimes severe enough to cause loss of consciousness as a neurologlycopenic symptom[16]. However, postprandial hypoglycemia following a gastrectomy usually occurs 1.5 to 3 h after food digestion[17]. The present case did not show characteristic postprandial hypoglycemia and the hypoglycemic symptoms improved during the two weeks after surgery, suggesting that this kind of hypoglycemia results from nesidioblastosis rather than from gastrectomy. Additionally, it is assumed that past history of a gastrectomy may have partly aggravated the hypoglycemia in addition to the effect of nesidioblastosis.

When clinical examinations including ASVS suggest nesidioblastosis, a partial pancreatectomy is usually performed. Even if a frozen biopsy confirms the diagnosis of nesidioblastosis, the extent of pancreatic resection remains questionable. A distal pancreatectomy which can control the symptoms of the majority of patients, is well tolerated, and does not induce endocrine or exocrine insufficiency. Recovery after a partial pancreatectomy can remove enough abnormal proliferative tissue to achieve normoglycemia[4,18]. However, some investigators recommend an initial near-total pancreatectomy. Such extensive resections lead to an increased risk of post-surgical diabetes and pancreatic insufficiency. It seems that the best recommendation is a 70%-80% pancreatectomy, administration of diazoxide when hypoglycemia persists post-operatively, and a more extensive resection when previous measures fail[19-21].

In summary, we report here a case of nesidioblastosis of a patient having a history of a subtotal gastrectomy based on a diagnosis of a gastric adenocarcinoma. ASVS can detect a hyperinsulinemic lesion of the distal pancreas. However, sporadic hypoglycemia may occur after surgery, and is refractory to medication. Further study is needed to improve its treatment.

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*CASE REPORT*

# Double ischemic ileal stenosis secondary to mesenteric injury after blunt abdominal trauma

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## Abstract

The authors describe a rare case in which blunt abdominal trauma resulted in mesenteric injury with delayed double ischemic ileal stenosis. Abdominal computed tomography demonstrated stenotic ileal loop with mural thickening. At surgery, a double stenotic bowel loop was found adjacent to a healed defect in the mesentery. Histological examination of the two resected segments showed fibrotic and ischemic lesions within the mesentery. Ischemic intestinal stenosis from mesenteric injury should be considered in the differential diagnosis in patients suffering from intestinal occlusion with a history of blunt abdominal trauma.



**Key words:** Blunt abdominal trauma; Mesenteric injury; Intestinal stenosis; Obstruction

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## INTRODUCTION

In the current treatment of blunt abdominal trauma, conservative management and observation with computed tomography (CT) have become the standard of care for the non-life threatening abdominal lesions. Ischemic intestinal stenosis has been described as a delayed rare complication of mesenteric blunt abdominal trauma. The authors present herein a case of post-traumatic mesenteric injury proved by histological examination, and with double bowel stenosis on CT scan and enteroclysis.

## CASE REPORT

A 35-year-old female was admitted after an automobile accident with sudden deceleration. She drove the car and carried the safety belt. The admission check-up showed a cranial trauma and multiple fractures, in particular of the two inferior limbs. She underwent contrast enhanced abdominal CT which showed moderate fluid in the peritoneal cavity attributed to recent bleeding, but no free air, and no parenchymatous, visceral or vascular lesions. She was transferred to the traumatologic unit for surgical osteosynthesis, after which she underwent revalidation treatment.

Six weeks later, she returned to the emergency department with abdominal pain. Altogether, she suffered from four painful abdominal crises with vomiting, described as acute, periumbilical cramps, spontaneously resoluting, compatible with subocclusion or König syndrome.

Abdominal CT showed two ileal loops with a thickened wall and narrowed lumen (Figure 1) adjacent to a mesenteric haziness and minimal fluid in the Douglas. There was no sign of absolute obstruction. An enteroclysis confirmed two intestinal stenosis with rigid ileal loops, one proximal and the other distal (Figure 2).

The patient underwent laparotomy. Two ileal lesions were observed, one located at 180 cm from Treitz's angle, the second one at 60 cm from the Bauhin's valve. Both were scarred ischemic post-traumatic lesions located in front of a mesenteric injury, with thickened ileal wall and narrowed lumen (Figure 3). The two stenosed ileal segments were resected and a latero-lateral anastomosis was performed. Recovery was uneventful. Pathological examination showed mucosal ulceration of the gut located in front of a mesenteric tear (Figure 4), and degenerative infiltration of the mesenteric small vessels, arterioles and veins (Figure 5).

## DISCUSSION

Some patients with traumatic intestinal lesion such as perforation or hemorrhage from disruption of mesenteric vessels or parenchymatous tears require immediate surgical intervention. Small and large bowel mesenteric injuries are found in approximately 13.5 % of all patients undergoing

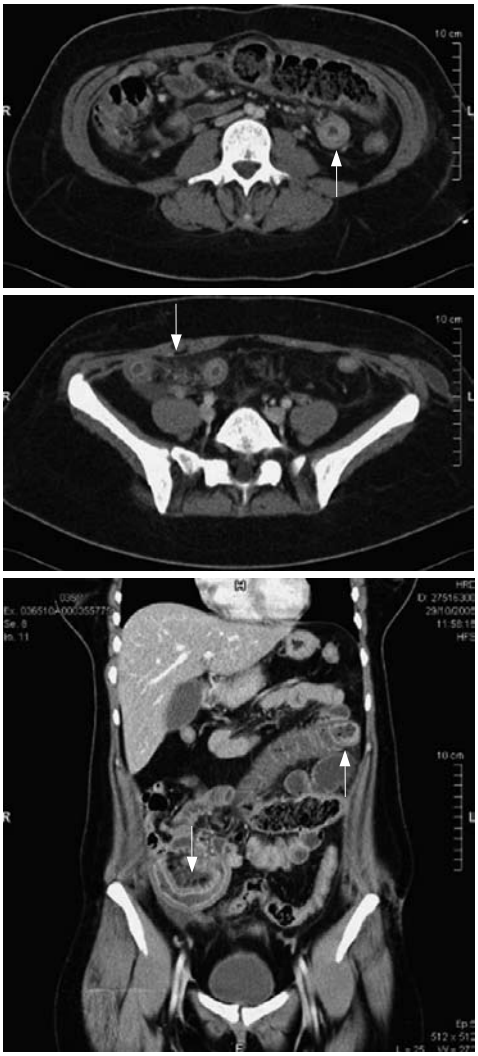


**Figure 1** **A**: Transversal abdominal computed tomography, demonstrating two thickened bowel loops with mesenteric haziness (arrows); **B**: Frontal abdominal computed tomography, demonstrating two thickened bowel loops with mesenteric haziness (arrows).

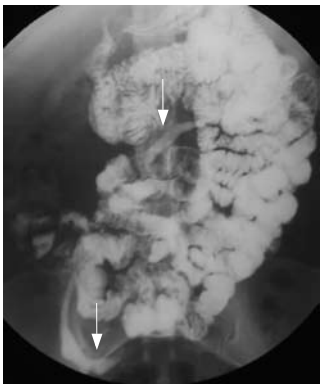

**Figure 2** Abdominal enteroclysis showing two intestinal strictures, one proximal, the other distal (arrows).

laparotomy after blunt abdominal trauma[1,2]. In this setting, small bowel injuries are more frequent than colonic injuries, probably due to the factors, including location and lack of redundancy, which prevents formation of closed loops[3]. Contrast enhanced CT should be performed early in patients with blunt abdominal trauma because most significant bowel and mesenteric injuries, as well as associated injuries to other abdominal viscera, may be

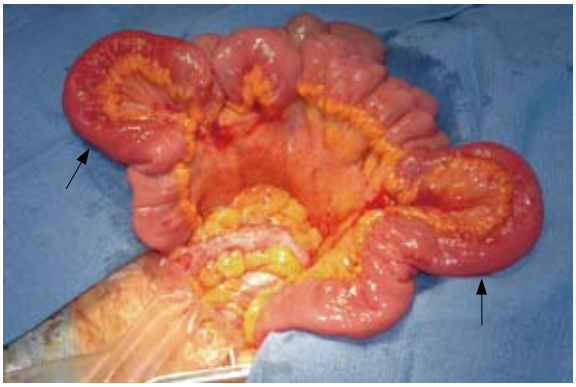

**Figure 3** Preoperative picture showing two thickened, fibrotic small bowel segments (arrows) in front of a scarred mesenteric tear.

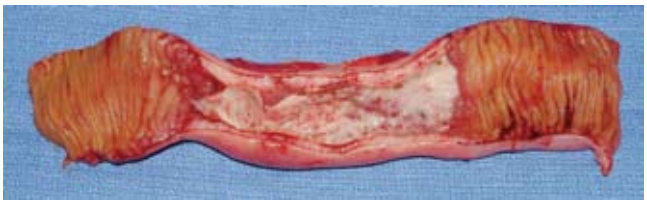

**Figure 4** Inflammation with mucosal ulceration.

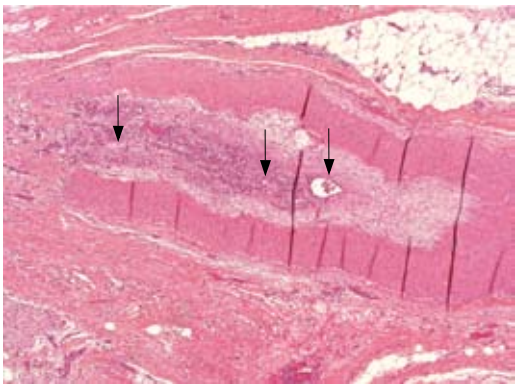

**Figure 5** Microscopic section of healed mesenteric injury showing vascular chronic lesion with a repermeabilised venous thrombosis (arrows).

reliably identified. The presence of a moderate to large volume of intra-peritoneal fluid without visible solid organ injury can be an important sign of mesenteric or bowel injury[1], similar to this patient.

On the other hand, mesenteric injury may not cause clinical manifestations[4]. Patients with blunt abdominal trauma are often managed without surgical intervention if there is no sign of bowel perforation or hypovolemic shock. As a result, mesenteric injuries might remain undiagnosed after blunt abdominal trauma.

Small bowel obstruction following blunt abdominal trauma is rare. Three possible causes have been suggested: small bowel perforation[5], localized bowel ischemia[1,4] and mesenteric injury[6]. Mesenteric vascular injury may induce chronic ischemia of the corresponding segment of small bowel, inducing secondary thickening of the bowel wall and intestinal occlusion[1,6]. Post-traumatic bowel lesions may also be found in the colon, mainly on the left

side, specifically the sigmoid colon[7]. The case described herein and its pathological report, further support the mesenteric injury theory of post-traumatic bowel strictures. Most published reports of post-traumatic bowel stenosis described one stricture of the small bowel[8]. The patient reported herein suffered from a double localisation of small bowel lesions, both treated by resection and anastomosis.

Under this particular circumstance, enteroclysis may demonstrate the localization of the stricture, showing a narrowed, rigid loop[9,10]. Abdominal CT may show a bowel wall thickening and a concentric narrowed intestinal lumen, or signs of small bowel obstruction with changed calibre of loops[1]. Although angiography may further demonstrate mesenteric vessel occlusion and provide additional information concerning mesenteric injury[9,11], the authors consider that abdominal CT may provide sufficient information to confirm the clinical suspicion of post-traumatic small bowel stricture and to indicate the need for surgical exploration. Laparotomy with resection of the stenosed segment and primary anastomosis is the appropriate treatment. Early recognition and intervention may prevent prolonged morbidity[10,12].

In summary, a rare case of intestinal occlusion secondary to double small bowel stricture due to abdominal blunt trauma and mesenteric injury was described. This diagnosis should be considered in all patients presenting with abdominal pain some weeks after blunt abdominal trauma.

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*CASE REPORT*

# Crohn's disease complicated by multiple stenoses and internal fistulas clinically mimicking small bowel endometriosis

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## Abstract

We report a 31-year-old woman with Crohn's disease complicated by multiple stenoses and internal fistulas clinically misdiagnosed as small bowell endometriosis, due to the patient's perimenstrual symptoms of mechanical subileus for 3 years; at first monthly, but later continuous, and gradually increasing in severity. We performed an exploratory laparotomy for small bowel obstruction, and found multiple ileal strictures and internal enteric fistulas. Because intraoperative findings were thought to indicate Crohn's disease, a right hemicolectomy and partial distal ileum resection were performed for obstructive Crohn's ileitis. Histopathology of the resected specimen revealed Crohn's disease without endometrial tissue. The patient made an uneventful recovery from this procedure and was discharged home 10 d post-operatively. The differential diagnosis of Crohn's diease with intestinal endometriosis may be difficult pre-operatively. The two entities share many overlapping clinical, radiological and pathological features. Nevertheless, when it is difficult to identify the cause of intestinal obstruction in a woman of child-bearing age with cyclical symptoms suggestive of small bowel endometriosis, Crohn's disease should be included in the differential diagnosis.



**Key words:** Crohn's disease; Endometriosis; Regional ileitis; Stricture; Internal fistula; Intestinal obstruction; Inflammatory bowel disease

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## INTRODUCTION

Endometriosis is a condition of unknown etiology in which proliferation of endometrial tissue occurs at extrauterine sites, including ovaries, fallopian tubes, peritoneum, and gastrointestinal tract[1]. It usually occurs between 30 and 40 years of age and remains active in some patients well into the postmenopausal period[2]. Four to 17% of menstruating women develop endometriosis[3]. Involvement of the intestine with endometrial implants complicates pelvic endometriosis in 17%-37% of cases[4]. Small bowel endometriosis may not be accompanied by pelvic endometriosis[5]. The most commonly affected gastrointestinal sites are the rectosigmoid colon, appendix, and ileum[6]. When the disease involves the small bowel, it usually has a benign course, but in rare circumstances, it may present as acute abdomen[7]. Invasive bowel endometriosis can present as bowel obstruction in an acute, chronic, or intermittent manner[8]. The major cause of obstruction is stricture formation and adhesions, which occasionally mimic Crohn's ileitis or a malignancy in their clinical presentation[9-12].

We report a case of Crohn's disease complicated by multiple strictures and internal fistulas clinically misdiagnosed as small bowell endometriosis, due to the patient's perimenstrual symptoms of mechanical subileus, which required surgical intervention with right hemicolectomy and partial distal ileum resection. To the best of our knowledge, this is the first reported case of complicated Crohn's disease clinically mimicking ileal endometriosis. Moreover, we have been unable to find in the medical literature any report of this particular presentation of complicated Crohn's ileitis.

## CASE REPORT

A 31-year-old woman was admitted to our hospital with perimenstrual lower and mid-abdominal pain irradiating to the back and lower abdominal fullness for 3 years, at first monthly, but later continuous, and gradually increasing in severity. She had a history of low-grade fever with chills, which occured monthly during her menstrual periods. The abdominal pain was crampy and associated with

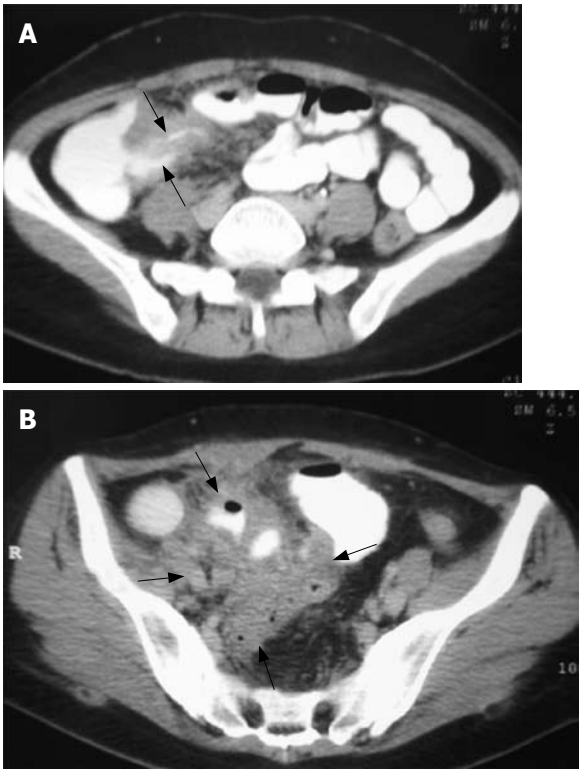


**Figure 1** Contrast-enhanced scan showing the mural thickening of terminal ileum (arrows) (**A**), and a complex, predominantly inflammatory mass of large size (6.4 cm × 6.1 cm) (arrows) (**B**).

borborygmi, anorexia, nausea and vomiting, without hematemesis or coffee-ground emesis. The patient had lost 10 kg in weight over the previous 6 mo, and was having two to three semiformed stools per day. She also gave a history of moderate dysmenorrhea and menorrhagia, but no dyspareunia. Her only medication was an oral contraceptive. She denied any history of smoking, alcohol consumption, or illicit drug use. The patient had no history of sexually transmitted disease. She had delivered a healthy baby. There was no family history of inflammatory bowel disease or endometriosis.

Despite no pathological evidence, her gynecologist at a women's health clinic had diagnosed her with small bowel endometriosis, based on interviews and her clinical course. Since only oral contraceptive therapy was started, the symptoms due to mechanical subileus had gradually improved. Although he had no pathological evidence, lack of response to oral contraceptive therapy had encouraged him to perform an exploratory laparotomy 1 mo before she was admitted to our institution for further evaluation and management. At Pfannenstiel transverse laparotomy, extensive adhesion formation and inflammation around the cecum, terminal ileum, sigmoid colon, uterus and the right adnexa were found. The gynecologist was only able to perform an incisional biopsy from the highly inflamed areas. Biopsy results showed only chronic inflammatory infiltrate and granulation tissue. The patient was then referred to us to identify the underlying pathology.

A physical examination on admission revealed a thin and wasted woman weighing 48 kg. The patient was hypotensive with a blood pressure of 95/65 mm Hg and a heart rate of 108 beats/min. The patient's temperature was 37.5℃. The abdomen was soft and distended with right-lower-quadrant tenderness to palpation and fullness. A Pfannenstiel scar was present, but there was no scar tenderness. There was no hepatosplenomegaly or lymphoadenopathy. She demonstrated no arthralgia. Skin rash and erythema nodosum were not found. Rectal examination was unremarkable. There was no perianal fistula. A pelvic examination revealed cervical motion tenderness and right adnexal tenderness. Results of laboratory studies on admission showed: white blood cell count, 28 000/mm$^3$ with 85.7% neutrophils; hemoglobin, 8.3 g/dL; hematocrit, 24.3%; erytrocyte sedimentation rate, 72 mm/h; C-reactive protein, 26.2 mg/dL; albumin, 3.2 mg/dL; β-human chorionic gonadotropin (β-hCG), 0.1 mIU/mL; carcinoembryonic antigen (CEA), 0.58 ng/mL; and cancer antigen (CA)125, 13.71 U/mL. Urinalysis was in the normal range. Stool studies for ova, parasites, cultures including *Yersinia enterocolitica* and acid-fast bacilli and *Clostridium difficile* toxin were negative. A plain abdominal roentgenogram was consistent with partial small bowel obstruction. Abdominal ultrasonography demonstrated the presence of an inflammatory mass surrounding the terminal ileum and/or appendix, wall thickening and absence of peristalsis in the small intestine, with a small amount of ascites. Abdominopelvic CT scanning was performed with oral and intravenous contrast enhancement. There was a narrowing of the terminal ileum and bowel-wall thickening (Figure 1A). Adjacent to the terminal ileum in the right lower quadrant, a complex predominantly inflammatory mass of large size (6.4 cm × 6.1 cm) was present (Figure 1B). The mass was surrounded by fat stranding, with obliteration of the adjacent psoas muscle fat planes. The appendix was not identified separately from the mass. Colonoscopy with random biopsies from the colon and rectum was unremarkable.

A presumptive diagnosis of mechanical small bowel obstruction was made. The patient was initially treated conservatively with nasogastric suction, intravenous fluids and medication, and responded to this treatment. However, after ingesting a small amount of food she again complained of abdominal pain, and plain radiography once more showed mechanical small bowel obstruction. After improvement with conservative measures and obtaining adequate informed consent, an exploratory laparotomy was performed. At surgery, the proximal small bowel was dilated and the colon was collapsed. There was a small amount of free fluid in the abdomen. No macroscopic evidence of endometriosis was noted at laparatomy. Multiple stenotic ileal loops, adhering firmly to each other, were wrapped as a mass lesion around the cecum, ascending colon, sigmoid colon, right adnexa and uterus. The adhesions were released, and ileal loops were freed from adjacent organs by meticulous sharp dissection. The serosa overlying these areas was congested and hyperemic. The right ureter was completely exposed. Four strictures were noted in the distal 40 cm of the terminal ileum, and three internal fistulas were detected between the terminal ileum and the cecum, between the terminal ileum and the adjacent loop of small bowel, and between the two loops of ileum (Figure 2A and B). The macroscopic appearance

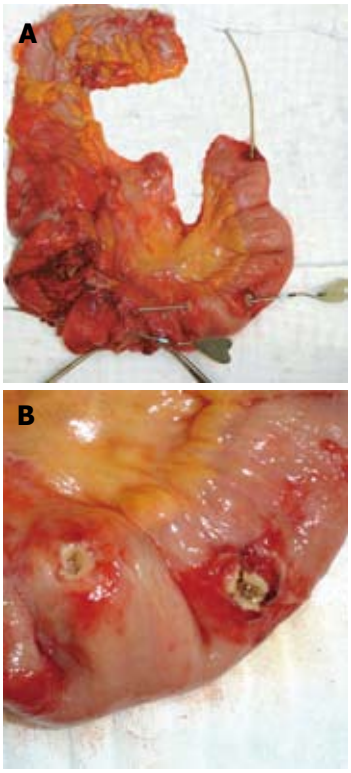


**Figure 2** Gross appearance of the resected specimen showing Crohn's ileitis with multiple fistulas probed with instruments (**A**), and ileal segment with two adjacent openings of an internal enteric fistula after separation of adhesions (**B**).

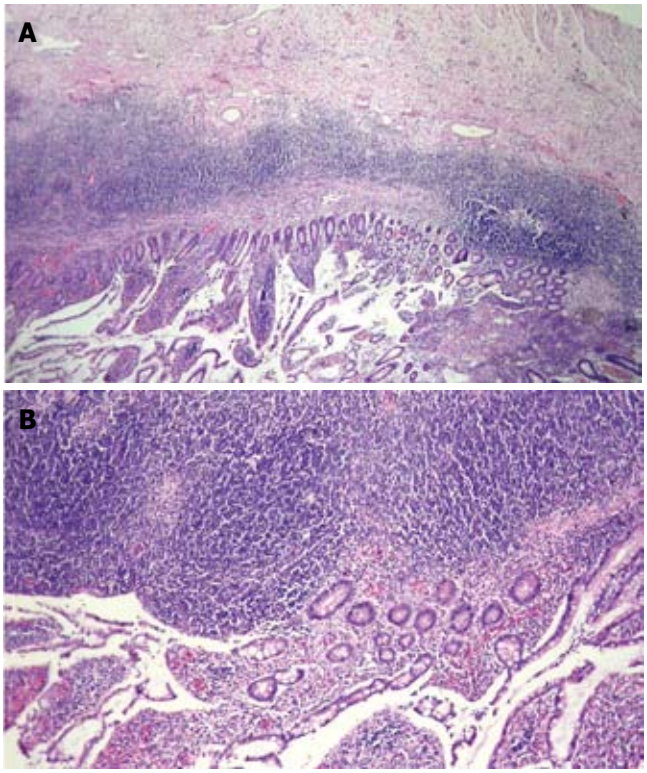


**Figure 3** Microscopic examination of the resected specimen revealed transmural inflammatory cell infiltration with crypt distortion (**A**), and transmural lymphoid aggregates (**B**) (HE, **A** × 10, **B** × 20).

was thought to indicate Crohn's disease, but in view of the close relationship of the ovaries, tubes and uterus, an immediate gynecological opinion was obtained. The on-call gynecology registrar did not consider the appearance to be due to primary gynecological pathology, and therefore, a right hemicolectomy and partial distal ileum resection was performed with an end-to-end ileocolonic anastomosis.

On opening the specimen, there was severe narrowing of the terminal ileum and bowel-wall thickening characteristic of Crohn's disease. Ulceration measuring 3 cm in diameter, with an irregular margin, was detected at the level of ileocecal valve. Multiple ulcerations, which ranged in sized from 0.5 cm to 2.5 cm in diameter, were also observed on the oral side of the terminal ileum and adjacent loops of small intestine. The mucosa was edematous, pale and marked with deep linear ulcers, and the bowel wall was thickened. Histological examination of the resected terminal ileum revealed marked ulcers and fissures, with transmural inflammatory cell infiltration (Figure 3A), lymphoid aggregates (Figure 3B), and fibrosis. No endometrial tissue was noted in any segment of the resected specimen. The patient made an uneventful recovery from this procedure and was discharged home 10 d post-operatively. Ileocolic resection led to rapid resolution of the symptoms. She has been asymptomatic for over 1 year after her surgery.

## DISCUSSION

Endometrosis is a common condition that occurs most frequently in the pelvis, in association with the ovaries, tubes and pelvic peritoneum, with related gynecological symptoms. The incidence of intestinal endometriosis varies between 3% and 37% of all cases of endometriosis, affecting the rectosigmoid colon in 50%-90%, rectovaginal septum in 10%-20%, cecum in 2%-5%, appendix in 3%-18% and small intestine in 2%-16%[13-17]. Small intestinal involvement is nearly always confined to the terminal ileum, which can be explained by its proximity to the tubes and ovaries. However, the correct diagnosis is often delayed because intestinal endometriosis may masquerade clinically as regional enteritis, ulcerative colitis, appendicitis, ischemic enteritis or colitis, diverticulitis, acute self-limited colitis, irritable bowel syndrome, ileocolonic intussusception, or a neoplasm such as colonic or small bowel cancer[18]. Therefore, many cases of bowel endometriosis have been clinically diagnosed based on interviews and response to hormone therapy.

This case demonstrated multiple strictures and internal fistulas that are frequently observed in complicated Crohn's disease by intestinal contrast, abdominal CT, or at surgery, but we could not make an accurate preoperative diagnosis. There were probably several major reasons for this misdiagnosis. First, our patient presented symptoms of mechanical subileus including intermittent abdominal pain, nausea, vomiting and weight loss, which occured monthly during her menstrual periods. Second, she lacked extraintestinal manifestations associated with Crohn's disease, including stomatitis, aphthous ulcer, colitic arthritis, pericholangitis, erythema nodosum, pyoderma gangrenosum, episcleritis, uveitis, nephrolithiasis, and hydronephrosis. Third, a histopathological diagnosis of the endoscopic biopsy specimen was reported to be negative for inflammatory bowel disease.

Endometriosis of the intestinal tract is a common

disorder that, when symptomatic, may be difficult to diagnose accurately. Intestinal endometriosis has a predilection for the terminal ileum, leads to obstruction, and affects women of reproductive age. Crohn's ileitis must be considered in the differential diagnosis of enteric endometriosis, as with our patient. Other conditions that may cause difficulty in young women with apparent terminal ileal disease include nodular terminal hyperplasia, *Yersinia* enterocolitis, tuberculous enteritis, lymphogranuloma, small bowel lymphoma, carcinoid tumors of the terminal ileum, primary adenocarcinoma of the small intestine, and Behcet's disease[9,10]. Many of these conditions will be diagnosed only with laparotomy.

The distinction of ileal endometriosis from Crohn's disease may be difficult pre-operatively. Indeed, there is a similarity between the two entities in terms of clinical presentation, symptomatology, radiological appearances, surgical and pathological findings. The nature of presentation is mainly dependent on the region of bowel affected for both ileal endometriosis and Crohn's disease. The ileum is the third most affected area, after the rectosigmoid colon and appendix, which is found to be involved in up to 7% of patients with intestinal endometriosis[19]. It usually involves the distal ileum and is limited to the serosa. More extensive and deeper involvement may produce intestinal obstruction by stenosis, kinking or adhesions. Fibrosis may cause puckering and kinking of the mucosa, which results in intestinal bleeding[20]. The presentation of ileal involvement may be an incidental mass found on barium enema or CT, or as a lead point for ileal intussusception. However, Crohn's disease almost invariably affects the gastrointestinal tract. Crohn's ileitis, affecting the ileum only, accounts for 30% of cases. There are three categories of disease presentation in Crohn's disease: stricturing, penetrating, and inflammatory (non-stricturing, non-penetrating disease)[21]. The clinical presentation of our patient with evidence of small bowel obstruction, multiple ileal strictures and internal fistulas at laparotomy suggested Crohn's ileitis, probably due to a combination of inflammatory mass in the right lower quadrant, proximal bowel distention, narrowing of the terminal ileum and bowel-wall thickening.

Endometriosis may present with a wide variety of symptoms that are more commonly associated with other diseases. Gastrointestinal endometriosis is suggested by dysmenorrhea, menorrhagia or perimenstrual symptoms. Intestinal involvement is usually only serosal, not associated with any intestinal symptoms, and only coincidentally noted at the time of open surgery or laparoscopy for gynecological symptoms. Frank intestinal symptoms are usually associated with intestinal obstruction[22]. While intestinal symptoms may occur during or be exacerbated by the menses, this association may not always be present. The symptoms coincide with menstruation in only 18%-40% of the cases in some series[23]. A recurring crampy lower or mid-abdominal pain is the most common presenting symptom for both enteric endometriosis and Crohn's disease. Moreover, right-sided or right-lower-quadrant abdominal pain often precipitates laparotomy or laparoscopy, possibly for the suspicion of appendicitis. Other symptoms that may occur in ileal endometriosis as well as Crohn's disease include diarrhea, constipation, nausea, vomiting, fever, anorexia, and weight loss. We initially considered ileal involvement by gastrointestinal endometriosis because of a history of the relationship between menstruation and presenting symptoms, and also previous surgery for misdiagnosis of enteric endometriosis in our patient.

Small bowel endometriosis is capable of producing both acute and chronic symptoms that can present a diagnostic and therapeutic challenge to the surgeon. Imaging studies may not provide an accurate diagnosis. The radiologic appearances of ileal endometriosis may be so similar to ileal Crohn's disease that a differential diagnosis is not possible. Plain abdominal X-ray or barium enema may be helpful. Contrast studies may demonstrate extrinsic bowel compression, abdominal mass, and bowel stenosis or angulation[24]. Typically, it appears as a long filling defect with tapering margins. Non-invasive radiological diagnostic aids are provided by CT or ultrasonography. Abdominal ultrasonography demonstrates the size, shape and location of lesions, but unequivocal diagnosis is rarely possible. In our patient, ultrasonography demonstrated the presence of an inflammatory mass in the right lower quadrant, and also wall thickening and the absence of peristalsis in the small intestine, with a small amount of ascites. CT is useful for evaluating the small bowel with enteroclysis protocols. The findings of enteric endometriosis on CT include one or more focal masses on the bowel wall or a lesion compressing the colon. Abdominal CT is especially useful when looking for intra-abdominal complications of Crohn's disease, such as abscesses, small bowel obstruction, or fistulas[25]. When thin-section abdominopelvic images with multidetector CT were obtained from our patient, there was a narrowing of the terminal ileum and bowel-wall thickening. It was not certain whether this was due to extrinsic compression or a primary intestinal lesion. Adjacent to the terminal ileum in the right lower quadrant, a complex, predominantly inflammatory mass was also detected on CT. Nevertheless, multiple stenotic ileal segments and small intestinal fistulas associated with Crohn's disease were not seen on CT slices, possibly due to extensive adhesion formation and inflammation. Therefore, we were not able to exclude enteric endometriosis according to the presented radiological findings pre-operatively.

Crohn's disease usually has a distinctive appearance operatively which is not to be confused with the findings in endometriosis, even if multiple site ileal endometriotic deposits are present[26]. Furthermore, in both diseases the surgical intention is limited resection of obstructing lesions, so there is no conflict of interest. An even more difficult, but more important operative distinction is malignant disease, and clearly this is relevant for cecal and ileal lesions, such as colonic or small bowel cancer. At operation, intestinal endometriosis appears as tumors having a glistening gray color on cross section[19]. In our patient, multiple stenotic ileal loops, which adhered firmly to each other, were wrapped as an inflammatory mass around the adjacent organs. The serosa overlying these areas was congested and hyperemic. Multiple ileal strictures

and internal enteric fistulas were detected at laparotomy. Intraoperative findings were thought to indicate Crohn's disease, hence a right hemicolectomy and partial distal ileum resection was the treatment of choice for obstructive Crohn's ileitis.

Both small bowel endometriosis and Crohn's disease are characterized grossly by patchy involvement of the small intestine with intervening, uninvolved skip areas of intestine. Moreover, both conditions may be transmural processes with marked chronic inflammatory changes resulting in the formation of strictures, adhesions, mucosal thickening, mural fibrosis, bowel angulation, stenosis, fibrosis and obstruction. Although transmural involvement by Crohn's disease is the result of chronic insult, strictures and masses in endometriosis result largely from profound smooth muscle hypertrophy around endometriotic foci present in the muscularis propria[27]. Other manifestations of Crohn's disease, such as perianal abscesses or fistulas, and inflammatory pseudotumors involving the cecum, terminal ileum and appendix, have been described in intestinal endometriosis[15]. In rare cases, endometriosis may cause deep fissures and even fistulous tracts, further mimicking regional enteritis. In the presented case, severe mural thickening of terminal ileum, with variable luminal stenosis or stricture formation and entero-enteric fistulas, were gross abnormalities. An approximately 40-cm segment of distal ileum, ileocecal valve and cecum had four strictures and three internal enteric fistulas, with associated serosal adhesions.

Endometriosis may cause many of the histological changes that are associated with Crohn's disease. The endometriotic deposists consist of well-defined endometrial glands embedded in a dense cytogenic stroma[29]. Previous studies have observed chronic mucosal changes ranging from focally branching surface crypts, mildly hyperplastic surface epithelium overlying endometriotic foci, hemorrhagic mottling, ulceration, ulceration with inflammation, occasional neutrophils and eosinophils, or a non-specific inflammatory cell infiltrate, to focal glandular architectural abnormality with edema and siderophages[30]. Chronic inflammatory infiltrates composed of lymphocytes and plasma cells surround the deep crypts, and transmural lymphoid aggregates are also seen. Superimposed acute changes with crypt abscesses and surface ulceration may be noted. The changes are focal in most cases and are flanked by normal, unremarkable mucosa[27]. However, histopathological features used to reach the diagnosis of Crohn's disease in the present case included both gross and microscopic features such as a discontinuous, asymmetric distribution of the lesions, patchy inflammation, ulceration, fissuring, transmural distribution of the inflammatory infiltrate and lymphoid aggregates.

To the best of our knowledge, there have been no other case reports of Crohn's disease complicated by multiple ileal strictures and internal enteric fistulas clinically mimicking small bowel endometriosis. Involvement of the intestinal tract with endometriosis may mimic clinically and pathologically a wide spectrum of diseases including infectious etiology, ischemic enteritis/colitis, inflammatory bowel disease, and neoplasms. The differential diagnosis of Crohn's disease with intestinal endometriosis may be difficult pre-operatively. The two entities share many overlapping clinical, radiological, and pathological features. Nevertheless, when it is difficult to identify the cause of intestinal obstruction in a woman of child-bearing age with cyclical symptoms suggestive of small bowel endometriosis, Crohn's disease should be included in the differential diagnosis.

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*CASE REPORT*

# Huge gastric disopyrobezoar: A case report and review of literatures

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## Abstract

We reported a case of huge gastric phytobezoar. The gastric phytobezoar was successfully removed through gastrotomy after two failed attempts in endoscopic fragmentation and removal. Disopyrobezoars could be treated either conservatively or surgically. Gastrotomy or laparoscopical management is recommended for the treatment of huge disopyrobezoars.



**Key words:** Phytobezoar; Disopyrobezoar; Stomach

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## INTRODUCTION

Phytobezoars are common bezoars in the gastrointestinal tract, including stomach and small intestine[1], but huge disopyrobezoars are rarely seen clinically. We report a case of huge disopyrobezoar (18 cm × 7.5 cm × 7cm), a kind of phytobezoar caused by persimmon, and to present our experience while reviewing the literature.

## CASE REPORT

A 47-year-old man presented with complaints of acute aggravated epigastric distention and pain for half a month after one year of persistent epigastric distention and acid regurgitation, which were uninfluenced by ingesting food. There were no obvious symptoms of gastrointestinal obstruction, e.g., nausea or vomiting. The patient had a long history of overindulgence of bitter persimmons from childhood without the history of gastric surgery.

On physical examination, a solid, movable ellipse mass could be palpated in the left epigastrium and the abdomen was soft. Gastroscopical examination showed a giant brown solid gastric bezoar of 15 cm × 7 cm in size (Figure 1), chronic superficial gastritis as well as two erosions at the corner of the stomach, which were pathologically proven to be chronic superficial active membraneous inflammation. Abdominal computerized tomography revealed a mass-like occupational lesion within the stomach (18 cm long and 7 cm in diameter) with air bubbles retained in its interstices and mottled appearance, compatible with the features of bezoars (Figure 2). The patient was then admitted to the department of gastroenterology of our hospital.

On the 3rd day after admission to hospital, endoscopy (*Fujinon EG 250 WR 5*, Fuji Photo Optical Company Ltd, Tokyo, Japan) was used to fragment the huge bezoar with a mouse-teeth clamp and snare, but it was unsuccessful to extract the bezoar despite the help of gastric lavage using sodium bicarbonate ($NaHCO_3$) because the bezoar was too hard and big. On the 4th d, another attempt of endoscopical fragmentation and extraction procedure also failed. On the 5th day, the patient was transferred to the department of general surgery for operation after two failed attempts in endoscopic fragmentation and removal of the gastric bezoar.

Gastrotomy was performed on the patient and a huge grey ellipse bezoar (18 cm × 7.5 cm × 7 cm) was removed from the gastric lumen (Figure 3). A piece of the bezoar obtained during endoscopy was analyzed by infrared spectroscopy, which revealed that 85% of them was composed of tannin and 10% was cellulose, therefore, the bezoar was considered as disopyrobezoar derived from bitter persimmon. The patient was discharged on the 7th postoperative day. During the 6-month follow-up, the patient did not complain of any discomfort and the two concomitant erosions healed uneventfully on medical therapy shown by another gastroscopy.

## DISCUSSION

Bezoars are classified according to their composition into phytobezoar, trichobezoar (hair), lactobezoar (concentrated

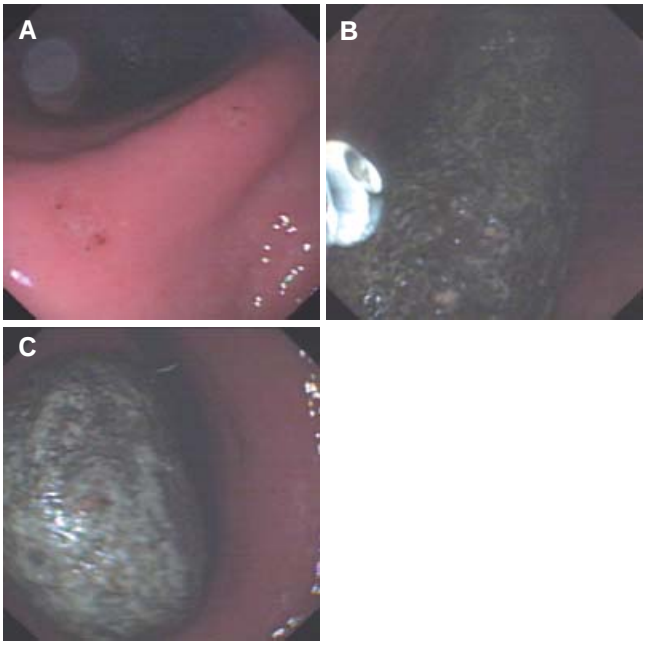


**Figure 1** **A**: A huge disopyrobezoar within the stomach shown by gastroscopy, with two erosions at the corner of stomach; **B** and **C**: A huge ellipse disopyrobezoar shown gastroscopically within the stomach.

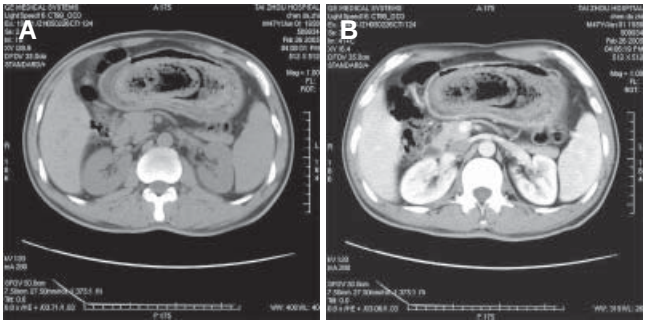


**Figure 2** **A**: Unenhanced CT scan showing a huge gastric mass with air bubbles and mottled appearance; **B**: Enhanced CT scan showing the same image as demonstrated on the unenhanced CT scan.

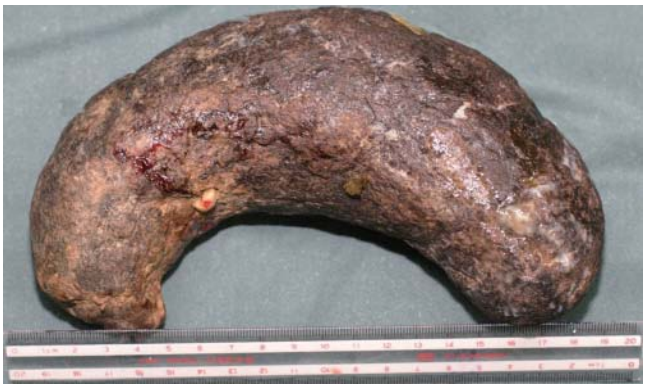

**Figure 3** Surgical specimen of gastric disopyrobezoar,18 cm long, 7.5 cm in diameter, with visible brownish persimmon remnants on its surface.

milk formulas), mixed medication bezoar and food bolus bezoar[2]. Phytobezoars are composed of indigestible cellulose, tannin and lignin derived from ingested vegetables and fruits[3].

A disopyrobezoar is a type of phytobezoar caused by persimmons, although it is an infrequent entity in clinic, and not rare in some countries[4]. Disopyrobezoars are generated by over-ingestion of unripe or astringent persimmons containing rich soluble tannin and shibuol especially in free stomach. In the presence of the dilute hydrochloric acid in the stomach, the tannin undergoes polymerization to a coagulum that includes cellulose, hemicellulose, and protein, which is the basis of the bezoar[5].

Disopyrobezoar formation is commonly associated with previous gastric surgery (truncal vagotomy plus pyloroplasty or subtotal gastrectomy plus gastroenterostomy), dental problems, poor mastication, and overindulgence of persimmon[4]. Gastric operations may reduce gastric motor activity and delay gastric emptying. Loss of pyloric function, gastric motility and hypoacidity plays an important role in the formation of disopyrobezoar[6]. Diabetes mellitus and hypothyroidism were also reported as predisposing factors of disopyrobezoar formation because they could delay gastric emptying[7]. Our patient had a long indulgence history of bitter persimmon intake, resulting in the formation of the huge disopyrobezoar.

Clinical manifestations vary with the location of disopyrobezoar from no symptom to acute abdominal syndrome, e.g., epigastric distention, abdominal pain and acid regurgitation. Gastric disopyrobezoars are sometimes associated with gastric ulcer formation. When located in small bowel, they often cause small bowel obstruction (SBO)[1,4], presenting with nausea, vomiting and abdominal distention. Major complications of disopyrobezoars include intestinal obstruction, gastric perforation, gastric ulcer and gastritis[5]. Abdominal pain (49%-100%), epigastric distress (80%), anorexia, vomiting and nausea (35%-78%), and SBO (94.73%) are the main clinical symptoms[8]. Feelings of fullness and dysphagia with weight loss and even gastrointestinal hemorrhage could also be seen[2]. When complicated with SBO, diminished peristaltic sounds, rebounding pain and tenderness, distention, vomiting and abdominal pain could be found clinically, elevated leukocyte count up to 28.000/mm$^3$ and fever could also be detected[5]. In this study, the patient presented epigastric distention, epigastric pain and acid regurgitation.

As for the radiographic diagnosis, about 50%-75% of the SBO cases could be diagnosed by plain abdominal radiography[9]. CT-scan could demonstrate a well-defined round mass which could be outlined by stomach or the bowel wall and present characteristic internal gas bubbles of bezoars[10]. Abdominal ultrasonography could suggest hyperechoic arclike surface and marked posterior acoustic shadow of the gastric bezoars within the lumen of stomach and small bowel, and barium study could demonstrate intraluminally filling defect as well as mottled appearance of the bezoar. Endoscopic investigations could almost show and confirm all of gastric bezoars[11].

The ultimate goal of the treatment of gastrointestinal disopyrobezoars is the removal of the lesion and prevention of recurrence. Disopyrobezoars can be treated by conservative modalities (gastric lavage, endoscopic

disruption, *etc*.) and conventional surgery as well as videolaparoscopic surgery[4,12]. But disopyrobezoars are often resistant to drug treatment because they are much harder than other kinds of phytobezoars, hence being usually removed endoscopically or surgically. Gastric lavage has been reported for the treatment of disopyrobezoars using $NaHCO_3$ which has a mucolytic effect, and penetration of $CO_2$ bubbles into the surface of bezoars could digest the fibres of concretion[6]; and most interestingly, a successful nasogastric Coca-Cola lavage treatment for gastric disopyrobezoars was reported[13].

The side effects of conservative treatment of bezoars are gastric ulcer, SBO, hyperosmolar natremia, hemorrhagic pulmonary edema, pharyngeal abscess, endotracheal tube obstruction, esophago-gastric iatrogenic injuries (including perforation, laceration, hematoma and ulceration), vocal cord damage and so on[11].

The first step of endoscopic procedure is to determine whether the pylorus appears anatomically normal and to verify the absence of duodenal stricture before fragmenting disopyrobezoars[4]. If a disopyrobezoar is not too large, it can be extracted by a basket or direct suction. If it is big and pylorus is normal, fragmentation can be performed with large polypectomy snare, electrosurgical knife, endoscopic laser destruction and electrohydraulic lithotripsy as well as extracorporeal shock wave lithotripsy[14]. Once the disopyrobezoar is fragmented, the patient can be treated by combined use of cellulase and metoclopramide, cellulase and papain, water jet and lavage with $NaHCO_3$ as well[4].

In conventional surgery, bezoar removal is commonly done by gastrotomy and/or enterotomy. If complicated with SBO, gastric perforation or gastric hemorrhage, the patients can be treated by gastric and/or intestinal resections. Our patient had a huge solid gastric disopyrobezoar that was extracted by gastrotomy after two failed attempts of bezoar removal by endoscopical fragmentation and dissolution by $NaHCO_3$ gastric lavage. Moreover, the laparoscopic approach may be the treatment of choice when surgery is indicated. When expertise is available, laparoscopy is safe and effective in the management of bezoar-induced SBO and yields superior postoperative outcomes when compared with conventional open approach[15].

In conclusion, disopyrobezoar is a kind of phytobezoar. Plain abdominal radiography, barium studies, ultrasonography, CT-scan and endoscopy are helpful in the diagnosis of disopyrobezoars, while endoscopy can confirm its diagnosis. Disopyrobezoars could be treated either conservatively or surgically. Gastrotomy or laparoscopical manipulation is recommended for the treatment of huge disopyrobezoars.

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*CASE REPORT*

# Anatomical variations of the cystic duct: Two case reports

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## Abstract

Anatomical variations of the cystic duct often occur and may be encountered during cholecystectomy. Knowledge of the variable anatomy of the cystic duct and cysticohepatic junction is important to avoid significant ductal injury in biliary surgery. Here, we present two unusual cases with an anomalous cystic duct, namely, low lateral insertion and narrow-winding of the cystic duct. The first case was a 64-year-old man with cholelithiasis and chronic cholecystitis. During surgery, the entrance of the cystic duct was misidentified as being short and leading into the right hepatic duct. Further exploration showed multiple calculi in the right and common hepatic ducts. Cholecystectomy was completed, followed by T-tube drainage of the common and right hepatic ducts. Postoperative T-tube cholangiography demonstrated that the two T tubes were respectively located in the cystic and common hepatic duct. Six weeks later, the retained stones in the distal choledochus were extracted by cholangioscopy through the sinus tract of the T-tube. The second case was a 41-year-old woman, in which, preoperative endoscopic retrograde cholangiopancreatography (ERCP) revealed a long cystic duct, with a narrow and curved-in lumen. The patient underwent open cholecystectomy. Both patients were cured. The authors propose that preoperative ERCP or magnetic resonance cholangiopancreatography (MRCP), and intraoperative cholangiography or cholangioscopy constitute a useful and safe procedure for determining anatomical variations of the cystic duct.



**Key words:** Cystic duct; Anatomical variations; Diagnosis; Cholecystectomy

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## INTRODUCTION

It is recognized that misidentification of normal anatomy, as well as the presence of anatomical variations, contributes to the occurrence of major postoperative complications, especially biliary injuries[1]. Such injuries can in turn cause significant morbidity and occasionally even mortality. Sound knowledge of the normal anatomy of the extrahepatic biliary tract, as well as the surgical implications[2], is thus essential to prevent these complications. Magnetic resonance cholangiopancreatography (MRCP) is a recently developed technique that allows non-invasive assessment of the biliary tree[3]. Anatomical variants of the cystic duct and cysticohepatic junction that may increase the risk of bile duct injury in biliary surgery are frequently identified with endoscopic retrograde cholangiopancreatography (ERCP), MRCP and percutaneous transhepatic cholangiography (PTC). We highlight two cases of anatomical variations of the cystic duct, in which the abnormality was found during surgery and subsequently confirmed by postoperative cholangiography and ERCP.

## CASE REPORT

### *Case 1*

A 64-year-old man was admitted for surgery, with features of acute cholecystitis: right upper quadrant pain, vomiting and fever. Examination revealed a temperature of 38℃ with tenderness in the right upper quadrant. He had previously been investigated for abdominal pain and had abdominal CT 4 years previously, which showed minimal intrahepatic ductal dilatation. Laboratory values showed a normal white cell count, total and direct biluribin was 24 μmol/L and 6 μmol/L respectively, alanine aminotransferase was 80 U/L, aspartate aminotransferase was 38 U/L, alkaline phosphatase was 301 U/L, and amylase was normal. Ultrasonography showed distension of the gallbladder with calculi and a thick wall associated with pericholecystic fluid. Cholelithiasis with chronic cholecystitis was diagnosed, and an open laparotomic cholecystectomy was planned. Upon laparatomy, the liver was normal and the gallbladder was thick-walled with minimal adhesion in Calot's triangle and surrounding

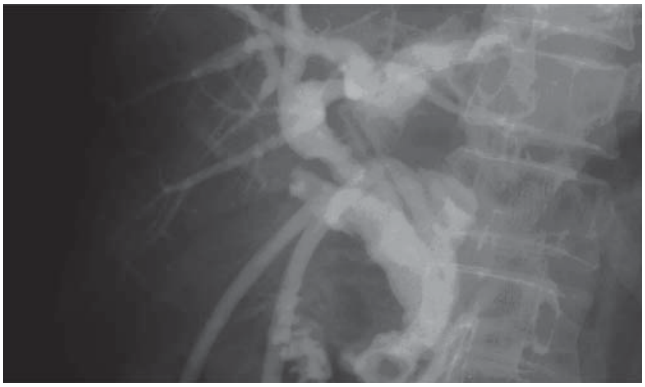

**Figure 1** The cystic and left-right hepatic ducts were comparatively long, and the cystic duct opening was located in the distal choledochus with retained stones. Two T tubes were respectively located in the cystic duct and in the common hepatic duct.

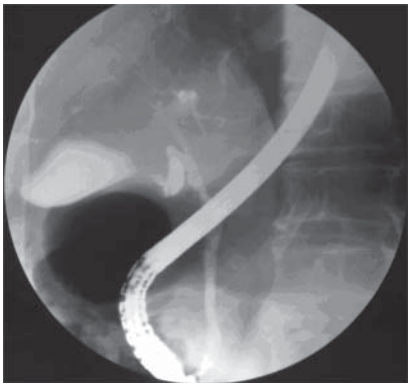

**Figure 2** ERCP showing a narrow-winding cystic duct.

tissues. On separation, the entering of cystic was misidentified as being short and entering the right hepatic duct. The cystic duct was clipped and divided. The cystic artery was then identified, clipped and divided. Further exploration showed multiple calculi in the right and common hepatic ducts. Cholecystectomy was completed, followed by T-tube drainage of the common and right hepatic ducts (for postoperative confirmation and documentation). The surgical procedure was followed by abdominal pain, and T-tube cholangiography was requested on postoperative d 14. Cholangiography through a T-tube showed that the cystic and right hepatic ducts were comparatively long. Long cystic duct with low medial insertion into common bile duct was made (Figure 1). The entire bile duct was identified and found to be intact. Six weeks later, the retained stones was extracted by cholangioscopy through the sinus tract of the T-tube. The patient was successfully cured.

### *Case 2*

A 41-year-old woman patient presented with 3-year discontinuous right upper quadrant pain that radiated into her back in keeping with biliary colic, sometimes accompanied with fever and nausea. The pain was aggravated with greasy food. In the course of the present illness, she had received medication for acid peptic disease and parasitic infestations, with no improvement. She had a similar history in the past. Physical examination revealed a soft, non-distended abdomen and no tenderness in the right upper quadrant, without peritoneal signs. Murphy's sign was negative. Her temperature was 37.5℃, and the rest of her vital signs were normal. Laboratory analysis of liver function was normal. US revealed no abnormalities. The patient was observed at rest with continuous gastrointestinal decompression and fluid infusion. Her symptoms improved significantly within 4 d of treatment. However, a few days later, she experienced right upper quadrant pain. ERCP was requested and revealed a long cystic duct with a narrow and in-curved lumen, which was well separated from the gallbladder. The rest of the entire biliary tract was normal without calculi (Figure 2). A narrow-winding cystic duct was created, followed by cholecystectomy. The surgical procedure was followed by an uneventful convalescence until discharge.

## DISCUSSION

Anatomical variations of the cystic duct are usually of no clinical significance, occurring in 18%-23% of cases[4]. However, unrecognized variant anatomy can be a source of confusion on imaging studies. In addition, the cystic duct may be involved in a wide variety of both primary and secondary disease processes. The rate of injury varies in the medical literature from 0% to 1%[5]. The following are some of the cystic duct variations found: (1) the cystic and common hepatic duct are in parallel; (2) low confluence of the cystic duct[2]; (3) insertion of the cystic duct in the left and right hepatic ducts, and bifurcation of the left and right hepatic ducts[2]; (4) anterior, posterior spiral types of insertion of the cystic duct on the left side of the common hepatic duct; (5) parahepatic duct insertion into the cystic duct; (6) absent or short cystic duct (length < 5 mm); (7) cystic duct hypertrophy, with a diameter > 5 mm; (8) double cystic duct[6,7]; (9) right hepatic duct emptying into the cystic duct[8]; and (10) hepaticocystic duct[9], a very rare congenital abnormality in which the common hepatic duct enters the gallbladder. The left, right, and common hepatic ducts are all defective, with the cystic duct draining the entire biliary system into the duodenum.

Multiple modalities permit depiction of the normal anatomy, as well as disease processes of the cystic duct, including CT, PTC, ERCP, intraoperative cholangiography and MRCP. Although visualization of the dilated cystic duct is possible with US and CT, the normal-caliber cystic duct may be difficult to detect with these techniques[10]. In our first case, CT demonstrated minimal intrahepatic ductal dilatation, but failed to show low insertion of the cystic duct, as was revealed by surgery. In this case, the low insertion of the cystic duct was misdiagnosed as gallbladder and bile duct calculi. However, in the second case, ERCP showed a long cystic duct with a narrow and in-curved lumen. An anomalous cystic duct was diagnosed before surgery. Anatomical variation is readily identified at ERCP. In clinical practice, if the patient presents with intermittent non-colic right upper abdominal pain, and ultrasound, CT and endoscopy eliminate choledocholithiasis, tumor and peptic ulcer, then a narrow-winding cystic duct should

be considered. ERCP is extremely helpful in diagnosis. Recent studies have demonstrated that MRCP may provide a non-invasive alternative to ERCP and PTC in diagnosis of anomalous cystic ducts[11]. Taourel and colleagues[12] evaluated the accuracy of MRCP in the diagnosis of anatomic variants of the biliary tree in 171 patients. MRCP demonstrated a cystic duct in 126 patients (74%), including low cystic duct insertion in 11 (9%) and a parallel course of the cystic and hepatic ducts in 31 patients (25%). These findings suggest that accurate preoperative assessment is very useful in providing a surgical treatment plan in addition to confirming diagnosis. During cholecystectomy, to avoid biliary tree injury, it is important to identify the common hepatic-cystic duct junction. Misidentification of the cystic duct can lead to postoperative complications. In particular, attention should pay to low medial insertion of the cystic duct because this anatomical variant may lead to misdiagnosis on imaging, and thus affect therapeutic intervention, as was seen in our first case.

A limited literature review of bile duct variation has shown that the aim of most surgeons is to identify whether there are bile duct stones. With respect to the accidental discovery of bile duct variation, it is not the nature of the variation itself but rather the existence of the bile duct variation that is the most important factor in the prevention of bile duct injury. Most injuries to the cystic duct usually occur when it runs parallel to the common bile duct and is encased in a common sheath, so that separation between the ducts is not readily apparent at surgery. T-tube placement in the cystic duct remnant is usually of no consequence; however, there may be a difficulty if retained common duct stones are present, and stone removal *via* the T-tube is attempted. In such cases, access to the bile duct is *via* a tract that enters the cystic duct, and manipulation and extraction must occur *via* the cystic duct across the valves of Heister. Stone extraction is more difficult or may be impossible *via* this route[13]. Suspicion should be raised if the cystic duct is of an unusually large calibre. Intraoperative cholangiography should be used in case of doubt and, in unusual circumstances, cholangiography can be performed *via* the gallbladder to aid in the identification of the cystic duct as well as the common bile duct.

In conclusion, the cystic duct may be involved in a variety of anatomical variations. Diagnostic accuracy relies on a clear understanding of the normal anatomy and anatomical variants of the cystic duct, and imaging features of calculous disease.

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*LETTERS TO THE EDITOR*

# Acute liver failure is frequent during heat stroke

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## Abstract

Acute liver failure (ALF) is relatively frequent during heat stroke (HS). This risk must be emphasized, because its incidence is higher than is usually thought. In a recent study by Weigand *et al*, two cases were reported in which liver failure was the leading symptom. We have confirmed their conclusion in a study of 25 cases of HS with ALF, compared with 25 other cases without ALF. Moreover, we observed that hypophosphatemia on admission could predict occurrence of ALF during HS. As for clinical and other biological parameters, phosphatemia should be monitored for at least 3 d in all cases of HS, even when it is thought to be mild.



**Key words:** Heat stroke; Hypophosphatemia; Liver failure

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## LETTER TO THE EDITOR

We were particularly interested in the excellent study by Weigand *et al*[1], who reported two cases of acute liver failure (ALF) after heat stroke (HS), one during a half marathon [exertional heat stroke (EHS)], the other classical, due to excessive environmental heat, and complicated by multi-organ failure. In addition to severe rhabdomyolysis, each patient was characterized by marked elevation of liver enzymes. Liver injury and ALF are known complications of HS[2]: serum alanine aminotransferase (ALAT) level usually rises within 30 min after HS and peaks within 3-4 d. The risk of ALF during HS must be emphasized, because its incidence appears to be higher than is indicated in this study. Irreversible ALF is rare and may require liver transplantation[3].

We have already reported some of our clinical data[4,5], from a cohort of 50 male subjects (which now consists of 110 cases); all investigated after EHS were confirmed by clinical and biological data, study of muscle metabolism by magnetic resonance spectrophotometry, muscle biopsy for pharmacodynamic tests, and pathology. Twenty-five of these consecutive patients (aged 25 ± 4 years) with EHS and ALF were compared with 25 other EHS patients, who were age-matched but without ALF (ALAT 3563 ± 1313 *vs* 590 ± 742 IU/L; factor V 30% ± 12% *vs* 66% ± 20%; $P < 0.001$ for each). ALF was defined as ALAT > 10 times the upper limit, and coagulopathy (factor V lower than 50%).

Age, body mass index, physical fitness, background, climatic conditions, drug and alcohol intake, clinical manifestations and laboratory findings were analyzed with reference to their prognostic significance in ALF. A logistic regression model was used. In 22 of 25 patients, ALAT level returned to normal within 10 d, but three patients died of ALF. Univariate analysis found that poor fitness ($P = 0.02$), hygrometry > 86% ($P = 0.03$), creatininemia > 160 μmol/L ($P < 0.001$) and hypophosphatemia < 0.5 mmol/L ($P < 0.001$) were significant predictors of ALF. In multivariate analysis, on admission, hypophosphatemia < 0.5 mmol/L was the only independent predictive factor of ALF (RR 3.8, 95% CI 1.1-6.2).

In conclusion, ALF is not uncommon in EHS of which we assert, as Weigand *et al*[1], that this one is an underestimated cause.

This ALF is strongly associated with early hypophosphatemia, of which a value < 0.5 mmol/L is predictive. However, there is no evidence that hypophosphatemia by itself causes important liver dysfunction[6]. Massive liver cell necrosis results from thermal shock, circulatory disruption, endotoxinemia (heat sepsis), high blood concentration of cytokines and acute-phase proteins.

Therefore, in EHS, measurement of phosphatemia, ALAT and factor V should be systematic on admission, and 3-4 d later. Phosphorus supply, usual in an intensive care unit, has not been evaluated in this situation.

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