# World Journal of Gastroenterology®

Volume 12 Number 30
August 14, 2006

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http: //www.wjgnet.com

ISSN 1007-9327 CN 14-1219/R Local Post Offices Code No. 82-261

World Journal of Gastroenterology
www.wjgnet.com
Volume 12
Number 30
Aug 14
2006

ISSN 1007-9327
CN 14-1219/R

WJG

World Journal of
Gastroenterology®

**Indexed and Abstracted in:**
Current Contents/Clinical Medicine and Science Citation Index-Expanded, Index Medicus, MEDLINE and PubMed, Chemical Abstracts, EMBASE/Excerpta Medica, Abstracts Journals, Nature Clinical Practice Gastroenterology and Hepatology, CAB Abstracts and Global Health.
ISI JCR 2003-2000 IF: 3.318, 2.532, 1.445 and 0.993.

**Volume 12 Number 30**
**August 14, 2006**

*World J Gastroenterol*
2006 August 14; 12(30): 4773-4936
**Online Submissions**
www.wjgnet.com/wjg/index.jsp
www.wjgnet.com
Printed on Acid-free Paper

A Weekly Journal of Gastroenterology and Hepatology

National Journal Award
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**COPY EDITOR FOR THIS ISSUE:** Ali Keshavarzian, MD

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PUBLISHED BY
The WJG Press

PRINTED BY
Printed in Beijing on acid-free paper by Beijing Kexin Printing House

 Print ISSN 1007-9327
CN 14-1219/R.

SPECIAL STATEMENT
All articles published in this journal represent the viewpoints of the authors except where indicated otherwise.

EDITORIAL OFFICE
*World Journal of Gastroenterology*,
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*World J Gastroenterol* 2006 August 14; 12(30): 4773-4783
***World Journal of Gastroenterology*** ISSN 1007-9327


*EDITORIAL*

# Current and future applications of *in vitro* magnetic resonance spectroscopy in hepatobiliary disease

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Supported by the Hammersmith Hospital Trustees Research Committee, the Medical Research Council, JEOL (UK) Ltd., Philips Medical Systems and the Higher Education Funding Council for England
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Received: 2006-01-11 Accepted: 2006-03-10

## Abstract

Nuclear magnetic resonance spectroscopy allows the study of cellular biochemistry and metabolism, both in the whole body *in vivo* and at higher magnetic field strengths *in vitro*. Since the technique is non-invasive and non-selective, magnetic resonance spectroscopy methodologies have been widely applied in biochemistry and medicine. *In vitro* magnetic resonance spectroscopy studies of cells, body fluids and tissues have been used in medical biochemistry to investigate pathophysiological processes and more recently, the technique has been used by physicians to determine disease abnormalities *in vivo*. This highlighted topic illustrates the potential of *in vitro* magnetic resonance spectroscopy in studying the hepatobiliary system. The role of *in vitro* proton and phosphorus magnetic resonance spectroscopy in the study of malignant and non-malignant liver disease and bile composition studies are discussed, particularly with reference to correlative *in vivo* whole-body magnetic resonance spectroscopy applications. In summary, magnetic resonance spectroscopy techniques can provide non-invasive biochemical information on disease severity and pointers to underlying pathophysiological processes. Magnetic resonance spectroscopy holds potential promise as a screening tool for disease biomarkers, as well as assessing therapeutic response.



**Key words:** Magnetic resonance spectroscopy; Liver; Hepatobiliary disease; Membrane metabolism

Cox IJ, Sharif A, Cobbold JFL, Thomas HC, Taylor-Robinson SD. Current and future applications of *in vitro* magnetic resonance spectroscopy in hepatobiliary disease. *World J Gastroenterol* 2006; 12(30): 4773-4783

http://www.wjgnet.com/1007-9327/12/4773.asp

## INTRODUCTION

The nuclear magnetic resonance (NMR) phenomenon was first reported in 1946[1] and has since been widely used by the scientific and medical communities. The 2003 Nobel Prize for Medicine was jointly awarded to Professor Sir Peter Mansfield and Professor Paul Lauterbur for their work on clinical NMR, and this serves to underline the impact on the medical community of the utility of this technique with all its diverse applications (www.nobel.se/medicine/laureates). In this highlight article, we explain the background to NMR and discuss various clinical studies that are relevant to the liver and the biliary system, with particular emphasis on *in vitro* applications that have analyzed body fluids, such as bile, or tissue obtained from liver biopsies.

NMR is a non-invasive and non-selective technique, allowing the study of molecular composition and structure. NMR forms the basis of magnetic resonance imaging (MRI) methodologies, which have been developed using whole body magnets. Spatial localization using magnetic field gradients has enabled very detailed images of the human body to be obtained. *In vivo* NMR spectroscopy studies can be performed as an adjunct to clinical MRI, as part of the same examination. Longitudinal studies can be readily undertaken using both of these MR applications. The term magnetic resonance spectroscopy (MRS) is used in this highlight topic to denote *in vivo* and *ex vivo* clinical NMR spectroscopy studies, and also encompasses *in vitro* NMR spectroscopy, performed in the laboratory at very high magnet field strengths.

## AN OVERVIEW OF MR SPECTROSCOPY

Although a detailed knowledge of the physical principles that govern NMR is not essential for appreciating the scope of NMR applications, it is often helpful to have some understanding of some of the basic concepts that underlie these methodologies. The NMR technique

exploits the behavior of atomic nuclei in an externally applied magnetic field. Magnetic resonance occurs because of the quantum mechanical property of "spin", which is intrinsic to certain atomic nuclei. Examples of such nuclei of clinical relevance include hydrogen-1 ($^{1}$H), carbon-13 ($^{13}$C), fluorine-19 ($^{19}$F), phosphorus-31 ($^{31}$P) and chlorine-35 ($^{35}$Cl). Such nuclei can be imagined to act like small bar magnets in a magnetic field by 'lining up' with or against the field, and can be excited by irradiation with non-ionizing radiofrequency (rf) energy. During relaxation following excitation, rf signals are generated which contain information regarding the magnetic environment experienced by each nucleus, and therefore about the molecules in which they exist. The resulting signals, the "free induction decay", are detected by a receiver coil and can be expressed as a frequency spectrum by the mathematical process of Fourier transformation. The relative frequency position of a metabolite signal, its chemical shift, is dependent on the locally experienced magnetic field and therefore the local chemical environment. Consequently, each nucleus type within a molecule has a characteristic chemical shift. Hydrogen-1 ($^{1}$H) and phosphorus-31 ($^{31}$P) are the two nuclei most commonly used for biological studies, as they are ubiquitous in nature ($^{1}$H = 99.985%, $^{31}$P = 100% natural abundance).

### *Metabolic significance of clinical MR spectroscopy*

Jacobson and colleagues[2] used MRS to examine the effects of hydration of DNA in 1954. Current investigations include both *in vitro* MRS studies on tissue samples or body fluids and *in vivo* whole-body MRS studies.

*In vitro* MRS studies include the analysis of body fluids (such as plasma, urine or bile), extracts of tissue, or small biopsy-sized specimens of intact tissue. *In vivo* MRS is more difficult to perform and is characterized by poorer resolution of metabolites than *in vitro* MRS. This is due to factors such as the lower magnetic field strengths used for *in vivo* MRS clinical studies and also to the effects of magnetic susceptibility and patient motion. The typical magnetic field strength is 1.5-3.0 Tesla (T) for clinical MRS studies and 11.7-18.8 T for *in vitro* MRS studies.

*In vitro* MRS can detect and characterize a range of metabolic components simultaneously, even if their chemical identities are unknown at the time of analysis. *In vitro* MRS studies therefore provide a comprehensive metabolic profile of the low molecular weight components in biofluids and tissues, reflecting levels of endogenous metabolites involved in key cellular pathways, which indicate the physiological and pathophysiological status. The technique can also provide a profile of exogenous agents, including xenobiotics and their metabolites, and give an indication as to their effects on endogenous compounds[3]. For such reasons, *in vitro* MRS has become one of the most successful and popular techniques for biofluid analysis over the past 10 years[4].

It is relevant to consider *in vitro* MRS applications to the liver in the context of metabolite findings from both *in vivo* $^{31}$P and $^{1}$H MRS studies of patients with liver disease, who have been examined using whole body MRI/MRS

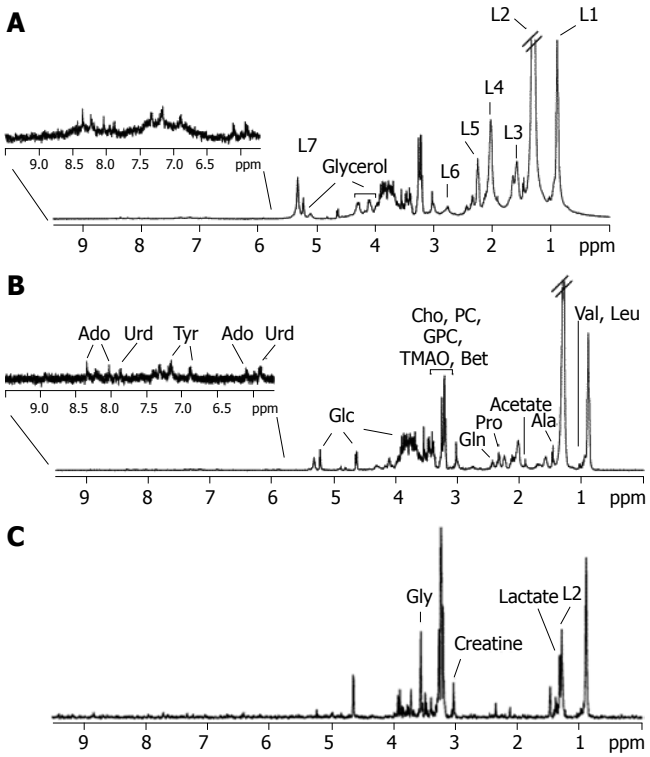


**Figure 1** 400-MHz $^{1}$H HR-MAS NMR spectra of a human liver biopsy sample (rotation rate 4 kHz). **A**: Standard 1D spectrum; **B**: Spin-echo (CPMG) spectrum; **C**: JRES f2 projection. L1: lipid $CH_3$; L2: lipid $(CH_2)n$; L3: lipid $CH_2CH_2CO$; L4: lipid $CH_2$-CH=CH; L5: lipid $CH_2CO$; L6: lipid CH=CH-$CH_2$-CH=CH; L7: lipid CH=CH; Cho: choline; PC: phosphocholine; GPC: glycerophosphocholine; TMAO: trimethylamine-N-oxide; Bet: betaine; Glc: glucose; Val: valine; Leu, leucine; Ala: alanine; Gln: glutamine; Gly: glycine; Tyr: tyrosine; Urd: uridine; Ado: adenosine. Reprinted with permission from Duarte *et al* Anal Chem 2005; 77: 5570-5578. Copyright (2005) American Chemical Society.

techniques. *In vivo* hepatic MRS studies have been primarily used in the research environment and have generally utilized $^{31}$P MRS[5], because $^{1}$H MRS is technically more challenging[6,7]. *In vivo* $^{31}$P MRS gives information on cell turnover and energy state and has been used to grade patients with chronic liver disease[8] and to aid in diagnosis and treatment response in patients with cancer[9]. On the other hand, *in vivo* $^{1}$H MRS allows quantification of intra-hepatocellular lipid (IHCL) levels, and therefore has been utilized recently to quantify the extent of steatosis in patients with fatty liver[10,11].

Representative hepatic $^{1}$H and $^{31}$P MR spectra are illustrated in Figures 1-4. *In vitro* $^{1}$H and $^{31}$P MR spectra of liver tissue are illustrated in Figures 1 and 3, and these can be compared and contrasted with *in vivo* hepatic $^{1}$H and $^{31}$P MR spectra illustrated in Figures 2 and 4. An *in vitro* $^{1}$H MR spectrum is dominated by contributions from water and IHCL. If the signal from water is suppressed using specific NMR methodologies, then more detailed contributions from IHCL can be observed along with resonances from choline-containing compounds (Cho) (Figures 1A-C). These data compare with the *in vivo* hepatic $^{1}$H MRS spectrum, which allows quantification of water and IHCL (Figure 2B).

*In vitro* $^{31}$P MR spectroscopy of liver tissue (Figure 3) allows characterization of resonances from phospholipid

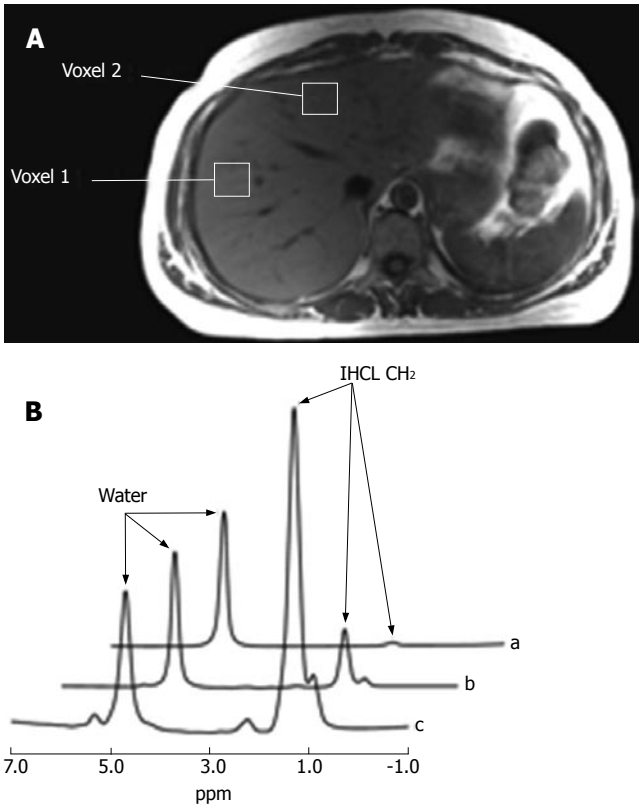


**Figure 2** **A**: A transverse image through the abdomen showing the two voxel positions used to study regional variation in hepatic fat content; **B**: Typical proton magnetic resonance liver spectra from three volunteers showing progressive degrees of fatty infiltration. Spectrum (a) shows a liver with minimal fatty infiltration (1.0%), spectrum (b) shows a liver with moderate fatty infiltration (10.2%), and spectrum (c) shows a liver with severe fatty infiltration (74.9%). Resonances from water and IHCL-$(CH_2)$n- can be clearly identified. Values refer to the peak area of the IHCL peak with reference to the water peak after correcting for $T_1$ and $T_2$. IHCL: intrahepatocellular lipids. Reproduced from Thomas *et al* Gut 2005; 54: 122-127, with permission from the BMJ Publishing Group.

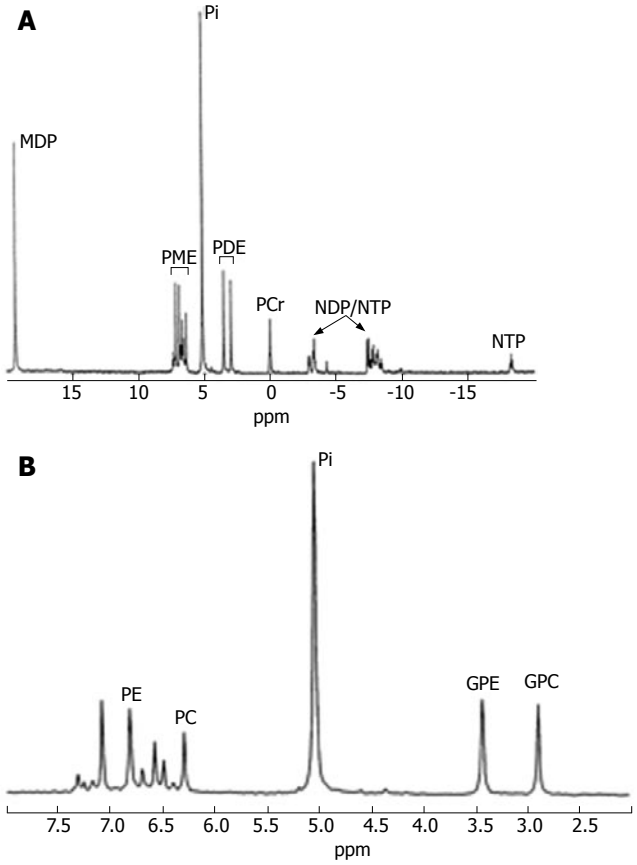


**Figure 3** Typical proton decoupled *in vitro* $^{31}$P MR spectrum of perchloric acid extracted normal liver tissue. **A**: Full spectrum; **B**: PME and PDE regions. PME: phosphomonoesters; PDE: phosphodiesters; NTP: nucleotide triphosphates; NDP: nucleotide diphosphate; PE: phosphoethanolamine; PC: phosphocholine; GPE: glycerophosphorylethanolamine; GPC: glycerophosphorylcholine; PCr: phosphocreatine; MDP: methylene diphosphonate. Reproduced from Taylor-Robinson *et al* Gut 1998; 42: 735-743, with permission from the BMJ Publishing Group.

cell membrane precursors, including phosphocholine (PC) and phosphoethanolamnine (PE), adenosine monophosphate (AMP) and glycolytic intermediates, such as glucose-6-phosphate. Cell membrane degradation products, including glycerophosphorylcholine (GPC) and glycerophosphorylethanolamine (GPE) and mobile phospholipids from the endoplasmic reticulum can also be identified. Depending on how the tissue is collected it is also possible to quantify contributions to the spectrum from inorganic phosphate and various nucleoside triphosphates (NTP). Such spectral resolution is difficult to achieve *in vivo*, and therefore a typical *in vivo* hepatic $^{31}$P MR spectrum (Figure 4) consists of six dominant, composite resonances, arising from: (1) the phosphomonoester region (PME), which are mainly cell membrane precursors and sugar phosphates; (2) inorganic phosphate (Pi); (3) phosphodiesters (PDE), which are mainly cell membrane degradation products, but also signal from mobile phospholipids contained in mitochondria and (4-6) three resonances from NTP, which are chiefly composed of adenosine triphosphate (ATP), but contain contributions from uridine triphosphate, guanosine triphosphate and cytosine triphosphate[12].

Changes in the PME and PDE metabolites indicate modifications in rates of cell membrane synthesis, breakdown, cell death and regeneration, associated with increasingly rapid cell growth, turnover and development[13]. Thus, these important phosphorus-containing molecules are intricately involved in the cellular processes linked to cellular destruction, turnover and malignant transformation.

MRS also provides important insights into dynamic metabolic changes in the diseased liver. However, unlike most liver function tests, the information obtained is not dependent on blood flow. Furthermore, most standard liver function tests also depend on measurements of plasma components, rather than assessing markers at the site of disease. Recent studies on hepatitis C suggest that the MRS technique may be useful in monitoring response to treatment with interferon and ribavirin[8].

### *Methodology for in vitro MR spectroscopy*

Sample preparation of body fluids requires routine clinical documentation to ensure that the relevant patient details are adequately controlled or accounted for, including time of sample collection and drug use history. It may be necessary to dilute or buffer the sample, or alternatively to document and account for any spectral changes relating to

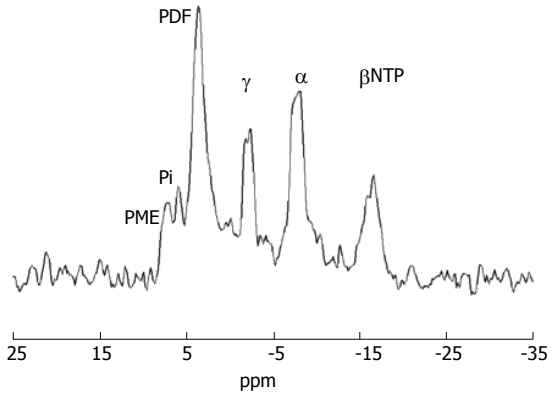


**Figure 4** Typical $^{31}$P magnetic resonance spectrum from the liver of a healthy volunteer (TR 10 000 ms). PME: phosphomonoester; Pi: inorganic phosphate; PDE: phosphodiester; NTP: nucleoside triphosphate; ppm: parts per million. Reproduced from Mullenbach *et al* Gut 2004; 54: 829-834, with permission from the BMJ Publishing Group.

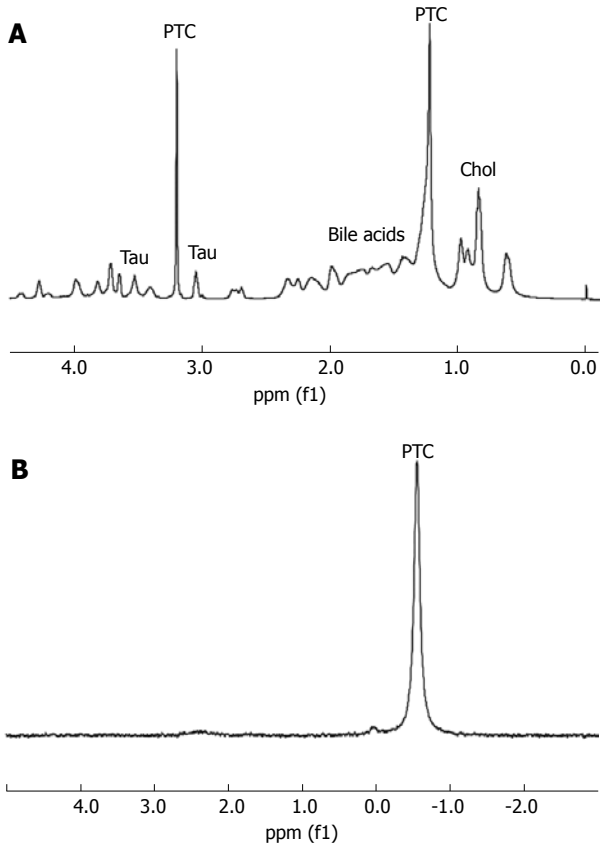


**Figure 5** **A**: $^{1}$H nuclear magnetic resonance spectrum of bile collected at laparoscopic cholecystectomy, after a history of recurrent cholecystitis. The spectrum has no contamination from contrast agents. The major peaks are assigned to phosphatidylcholine (PTC), bile acids, cholesterol (Chol), taurine (Tau) and the reference, sodium trimethylsilyl-[$^{2}H_4$] propionate (TSP); **B**: Corresponding $^{31}$P nuclear magnetic resonance spectrum. The major peak is assigned to PTC. ppm, Parts per million. Reproduced from Khan SA, Cox IJ, Thillainayagam AV, Bansi DS, Thomas HC, Taylor-Robinson SD. Proton and phosphorus-31 nuclear magnetic resonance spectroscopy of human bile in hepatopancreaticobiliary cancer. Eur J Gastroenterol Hepatol 2005; 17: 733-738, with permission from Lippincott Williams & Wilkins, Inc.

pH or concentration.

Sample storage should not cause a change in metabolite profile. Therefore storage temperature and conditions should be considered, and whether a change in sample temperature alters metabolite profile, for example during a freeze-thaw process[14,15].

Immediately prior to data collection, a known amount of a NMR standard can be added for NMR referencing and quantification. For example, sodium trimethylsilyl-[$^{2}H_4$] propionate (TSP) is routinely used as a reference compound for $^{1}$H MRS application. If the sample is required to be uncontaminated by any NMR reference compound for any further analysis, then either a reference compound can be placed in a capillary tube within the sample or an external standard is used.

The temperature of NMR data acquisition is under spectrometer control, so the sample temperature should be specified and constant throughout the study protocol. Many studies are performed at 25℃, but it may be necessary to study intact cells at lower temperatures to minimize the effects of ongoing cellular changes.

MR data acquisition includes adjusting the magnetic field homogeneity within the sample (a process known as 'shimming'). Data averaging is generally required, so as to improve the signal-to-noise ratio in the spectrum. Data can be acquired using pulse-collection methods or one- or two-dimensional NMR data collection protocols[16]. The water signal is generally suppressed using saturation techniques and/or spin-echo methods, in order to more readily observe the metabolite signals[17]. Intact tissue specimens may be analyzed using conventional solution MR spectroscopy techniques, by simply placing the biopsy sample within a NMR tube or by chemically extracting aqueous or lipid soluble fractions of the tissues. Studying intact tissue specimens directly within a conventional NMR tube has the disadvantage that magnetic field inhomogeneities within the sample do limit the achievable spectral resolution, but has the advantage that sample preparation is minimal and the sample remains intact and available for further analysis by other complementary techniques. Tissue extract studies have the advantage that the sample which is finally available for MRS study that has taken place after the extraction process, has become homogeneous so that the spectral peaks are well defined. However, the obvious disadvantages include the total destruction of the sample, the additional influence of metabolite solubilities in the extract medium and the relatively large amount of tissue required (upwards of 100 mg). The recently introduced method of magic angle spinning (MAS) MRS can overcome the limitations of both of the above techniques[18].

### *Magic angle spinning (MAS) MRS*

The line-broadening effects in an intact semi-solid are caused by constraints on molecular motion from cellular architecture, chemical shift anisotropy and dipolar couplings, and these can be reduced by spinning the sample at an angle of 54.7° to the main magnetic field, known as the 'magic angle'. While such MAS MRS techniques have been used for many years in the NMR spectroscopy study of true solids, MAS MRS has only recently been applied to the study of intact tissue[18]. A distinct advantage is that a MAS MR spectrum can be obtained from as little as 2

mg tissue and the tissue remains sufficiently intact to allow subsequent histological or genetic analysis[19].

As in conventional MRS studies, accurate determination of metabolite concentrations is pivotal to the success of tissue MAS MRS studies. A number of factors need to be considered for metabolite quantitation in MAS MRS, including possible leakage of metabolites during washing of biopsies prior to analysis[15], temperature at which the spectrum is acquired and appropriate choice of reference standard. The question of how to define a standard for quantitation is particularly important. For example the tissue water signal measured from a proton spectrum without water presaturation, may be used as an internal standard. Silicone rubber, for example, provides a useful external standard. Alternatively peak area ratios can be used to compare different spectra, but this has limitations as with *in vivo* MRS studies. For example, not only can changes in absolute concentrations be missed by the use of metabolite ratios, but an increase in the area of one peak can have the same result on the area ratio as a decrease in the other. Therefore, relying on the ratio of signal areas may mask metabolite differences inherent in the spectra.

## APPLICATIONS OF *IN VITRO* MR SPECTROSCOPY IN LIVER DISEASE

### Cellular bioenergetics

Owing to the ubiquity of phosphorus-containing moieties in energy metabolism, $^{31}$P MRS has been used to assess energy states in living systems[20]. However, the rapid degradation of phosphorylated nucleotide intermediates *ex vivo*, such as adenosine triphosphate (ATP) and adenosine diphosphate (ADP), has made snap-shot *in vitro* assessment difficult, although efforts have been made to minimize the ischemic time. Oxidation of these metabolites can occur at harvesting, snap freezing, thawing and during tissue extraction. In order to obtain metabolically useful information, the samples should be snap-frozen as soon as possible. In order to eliminate the need for the extraction of aqueous and lipid fractions, MAS MRS techniques could be used but data collection may need to be done at 4℃ to minimise further metabolic reactions during data collection.

An alternative methodology to assess bioenergetics is to use an *in vitro* perfusion system. Applications have included the effects of normoxia and anoxia in rat livers[21], the response to cyanide intoxication[22] and the response of damaged liver to fructose loading[23].

### Tumours

$^{31}$P and $^{1}$H MR spectra from aqueous soluble metabolites in extract of liver tumours of different types (hepatocellular carcinoma, colorectal metastases and secondary lung carcinoma) have been compared with samples from normal liver tissue and from histologically normal liver of the tumour host[24]. A significant increase in phosphoethanolamine (PER), phosphocholine (PC), taurine and lactate has been reported, with a reduction in GPE glycerophosphorylethanolamine (GPE) and glycerophosphorylcholine (GPC) in tumours, compared to normal liver controls[24]. In addition, the spectral changes identified by $^{31}$P MRS in tumour tissue have been seen at a lesser magnitude in the tumour host tissues[24].

Hepatic $^{31}$P MRS has been translated to the *in vivo* setting by Cox and colleagues and others[25]. The observed hepatic *in vivo* MR spectrum demonstrated a significantly raised PME/PDE ratio in tumour compared to controls. These peaks are thought to comprise increased PE and PC, and decreased GPE and GPC respectively, as elicited by *in vitro* analysis of tissue extract[24].

*In vitro* $^{1}$H MRS in conjunction with a statistical classification strategy has been used by Soper and colleagues to differentiate histologically normal liver, cirrhotic nodules and hepatocellular carcinoma[26]. Multivariate and pattern recognition techniques enable all data points to be incorporated into the analysis, no matter whether the metabolites comprising the spectral peaks have been identified. In this study, cirrhotic nodules were distinguished from hepatocellular carcinoma in 98% of cases[26].

### Chronic liver disease

Initial hepatic MRS studies of chronic liver disease focused on cirrhosis of varying aetiologies. Human livers with histologically proven cirrhosis have been assessed using *in vitro* $^{31}$P NMR spectroscopy[7]. Spectra from these patients with end-stage liver disease, all of whom had tissue obtained at the time of liver transplantation, showed significant elevations in phosphoethanolamine (PE) and phosphocholine (PC) and significant reductions in glycerophosphorylethanolamine (GPE) and glycerophosphorylcholine (GPC), when spectra were compared with those from histologically normal livers. Whether the patient had compensated or decompensated disease did not significantly alter the spectra obtained. Further work[27] by the same authors correlated such *in vitro* findings with $^{31}$P MRS *in vivo* and these studies suggest a potential clinical utility for *in vivo* MRS as an addendum to a standard MRI protocol in staging of the end-stage liver disease in patients that are being imaged for surveillance of hepatocellular carcinomas.

More recently, *in vivo* hepatic $^{31}$P MRS has been used to stratify inflammation and fibrosis caused by hepatitis C virus, compared to histological staging from standard liver biopsy[8]. The PME/PDE ratio was found to be elevated in mild, moderate/severe fibrosis and cirrhosis compared to normal, healthy volunteers. These changes have been thought to represent increased cell membrane turnover, although differences between means for each group were statistically significant, there was some overlap between the patient groups[8]. While histology remains the gold-standard, sampling error is inherent in the technique and it has been postulated that hepatic MRS could potentially provide a more accurate representation of the disease process, owing to the fact that it provides metabolite information from most of the liver[8]. Further studies directly correlating *in vivo* and *in vitro* MRS findings are awaited in this context.

While *in vivo* hepatic $^{31}$P MRS has been shown to correlate closely with disease severity in hepatitis C, its availability as a technique is not widespread. *In vivo* hepatic $^{1}$H MR spectroscopy would be more generally applicable on standard MRI scanners. Cho and colleagues have used this

technique to stratify chronic hepatitis compared to histology[28]. They were able to detect differences between groups, but the signal-to-noise ratio was low and assignment of the spectral peaks is open to debate[29]. A rational approach would be to identify the change in spectral peaks *in vitro*, using $^{1}$H MAS MRS, prior to translating the technique to the *in vivo* setting. With this in mind, Martínez-Granados and colleagues performed $^{1}$H MAS MRS on needle biopsies of liver tissue from 16 patients with chronic hepatitis or cirrhosis and one specimen from autopsy, nominated a "normal" control. High quality spectra were obtained and a number of metabolites assigned to previously unidentified peaks. Data collection was carried out at 4℃ to minimize tissue degradation. Differences in signal intensities between disease states and "normal" liver were noted, particularly increases in mobile fatty acids and glycogen in the former. However, the "normal" liver was harvested 24 h post mortem, so assumptions of metabolic normality are questionable[30].

### Hepatic steatosis

The intrahepatocellular lipid (IHCL) component of lipid extracts from steatotic liver specimens as assessed by $^{1}$H MR spectroscopy has been calibrated with *in vivo* MR spectroscopy measurements to estimate the lipid volume fraction in fatty liver disease[31]. There is an agreement with CT estimations but histomorphometry appears to underestimate the fat volume in samples. Szczepaniak and colleagues used localised *in vivo* $^{1}$H MRS in 2349 patients to assess the hepatic triglyceride content and estimated the prevalence of hepatic steatosis in the study group as 33.6%[32]. Further MRI studies *in vivo* have demonstrated a close relation between hepatic steatosis and body adiposity, and a close correlation between MRI estimation of adiposity and histological assessment in two of these patients[11]. Although studies have shown a close association between *in vivo* estimates and biopsies, *in vitro* MRS assessment of lipid content in liver biopsies by MAS MRS would allow direct comparison with histology, reducing the effect of sampling error[33].

### Orthotopic liver transplantation

Liver transplantation is used as a definitive therapy for acute hepatic failure, severe chronic liver disease and some cases of hepatocellular carcinoma. Demand for donor livers is high and suboptimal specimens may be used. At present there is no reliable non-invasive method to assess the viability of livers between organ harvesting and implantation. A program of research to address these problems demonstrates translational techniques from animal models *in vitro* to the clinical environment.

*In vitro* and *ex vivo* MRS studies of animals have established a model, which follows human organ harvesting and storage protocols[34,35]. Rapid reductions in ATP levels, readily measured by *ex vivo* $^{31}$P MRS have been seen in a pig model compared to rodent models and levels could be replenished by hypothermic reperfusion[34]. Now that MR probes have been designed to fit within organ retrieval boxes, clinical studies of organs within a transplant program are required[36].

In a preliminary study, Duarte and colleagues used *in vitro* $^{1}$H MAS MRS to assess biopsies taken at three time-points from six livers, before removal from donors, during cold perfusion and following implantation into the recipient[33]. The biopsies with the highest concentration of peaks reflecting fatty acyl chain (triglyceride) resonances were also identified as those also estimated to have the highest fat content on histological analysis. Other metabolites were identified, including glycerophosphocholine (GPC), which were reported to decrease from pre- to post-transplant[33]. However, in further studies such spectral changes need to be correlated with a range of clinical endpoints, including the premorbid clinical history of the liver donor, the pre-transplantation clinical history and nutritional status of the recipient, the subsequent post-transplantation liver function tests, pre-and post-transplantation indices of nutrition in the recipient and ultimately, the final clinical outcome.

Moving towards using MRS as a non-invasive methods for assessing graft dysfunction following transplantation, Taylor-Robinson and colleagues used correlative *in vitro* MRS of liver biopsy material and *in vivo* whole-body clinical hepatic $^{31}$P MRS to examine chronic ductopenic rejection of human liver allografts and noted an increased PME/NTP metabolite ratio reflecting associated altered phospholipid metabolism[37]. The study also included electron microscopy of liver tissue and the alteration in the phospholipid component was judged to be related to a change in biliary phospholipid excretion in these cholestatic patients[37]. Such an increase in the *in vivo* PDE resonance, seen in the *in vivo* hepatic MR spectrum is not a specific finding in patients with chronic allograft rejection, because a similar, albeit less marked change has been found in patients with primary biliary cirrhosis and obstetric cholestasis[38,39].

### Iron studies

Iron overload may be a result of iatrogenic causes such as multiple blood transfusions in beta-thalassaemia, or of metabolic causes such as hereditary haemochromatosis, and is commonly associated with liver dysfunction. At the current time, hepatic iron stores are still usually estimated using liver biopsy with the associated risks that this procedure carries, but are readily studied by MR, since paramagnetic iron compounds cause magnetic inhomogeneities, which shorten the nuclear relaxation time[40,41]. However, imaging is difficult on account of rapid relaxation caused by these iron moieties. In addition, use of different MR sequences is required and equipment may require recalibration. Relaxometry allows measurement of relaxation times and provides information as to iron-proton interactions. An *in vitro* approach allows direct quantification of iron following MR analysis[42]. *In vivo*, Wang and colleagues demonstrated single voxel MRS measurement of $T_2$ in liver iron overload that correlates strongly with iron quantification from biopsy and overcomes the difficulty of lack of detectable signals in conventional MRI[43]. Furthermore, Gandon and colleagues have proposed the use of a liver to muscle intensity ratio, which is transferable between equipment and sequences[44].

### Gene therapy

Gene therapy offers the opportunity to replace defective genes in phenotypically abnormal tissue with recombinant genes. Integration into the host cell genome allows expression of the desired protein. However, methods of conveying the gene to the required location and monitoring delivery and expression are required. Viral vectors and non-viral means such as naked DNA, liposomes and molecular conjugates have all been used. Expression varies with time and between tissues. MR techniques using MR spectroscopy and/or MR imaging, offer non-invasive methods of monitoring expression[45,46]. As in drug monitoring techniques where a MR detectable moiety or metabolite is linked to the active drug, genes expressing markers may be combined with the therapeutic gene. Phosphoarginine produced by the enzyme arginine kinase in *Drosophila,* but not present in mammalian skeletal muscle, is expressed in mouse skeletal muscle and detected by $^{31}$P MRS following injection of an adenovirus vector[47]. The expression in neonatal mice could continue for up to eight months. This demonstrates elegantly the principle of 'marker metabolite' using xenogenetic material. This principle has been demonstrated *in vitro* using a hepatocyte cell line[48]. Hepatocytes do not express creatinine kinase so phosphocreatine has not been seen on the hepatic MR spectrum. Integration of the cytoplasmic creatine kinase into hepatocytes *in vitro* could lead to detection of phosphocreatine by $^{31}$P MRS, raising the possibility that combined with a hepatotropic gene delivery system, gene expression may be monitored *in vivo* through a MR-visible marker.

## APPLICATIONS OF *IN VITRO* NMR SPECTROSCOPY IN BILIARY DISEASE

### Introduction

Bile is predominantly an aqueous solution containing numerous constituents. Bile acids (BA), phosphatidylcholine (PTC) and cholesterol are the predominant lipid components of bile. Other components include electrolytes, organic anions (bilirubin), plasma proteins, hepatocyte proteins, peptides and amino acids, nucleotides, heavy metals and vitamins, xenobiotics and toxins[49]. In health the concentration of these biliary constituents is tightly controlled.

Primary bile acids such as chenodeoxycholic acid (CDCA) and cholic acid (CA), and secondary bile acids such as lithocholic acid (LCA) and deoxycholic acid (DOCA) are conjugated with the amino acids taurine and glycine and secreted as sodium or potassium salts into the biliary canaliculi via the ABC biliary transporter proteins[50]. Phosphatidylcholine, the predominant biliary phospholipid, is synthesized in hepatocytes and transported into the biliary canaliculi by the flippase multidrug resistant protein 3 (MDR3)[51]. Its main function is to form mixed micelles with primary and secondary bile acids and cholesterol essential for the emulsification of fats. More recently it has been shown to be cytoprotective to the biliary epithelium[52]. Cholesterol is solubilized by the formation of vesicles with PTC or mixed micelles with bile salts and PTC.

Sampling of bile for diagnostic purposes has become a common clinical practice since the introduction of endoscopic retrograde cholangiopancreatography (ERCP). Bile research has advanced in the last decade mainly as a consequence of the alarming rise in incidence of biliary tract cancer (cholangiocarcinoma)[53-55].

Advanced cytological techniques such as digital image analysis and fluoresence *in situ* hybridization (FISH) of bile are being used to improve the diagnostic accuracy of cholangiocarcinoma in certain experimental units[56]. More recently proteomic analysis of cholangiocarcinoma bile has identified 87 unique proteins including several novel proteins whose functions are unknown and a large number of proteins not previously described in bile[57]. Advances in molecular research and imaging technologies have vastly improved the diagnostic sensitivity and specificity in this area but there is still a clear need to identify novel, highly sensitive and specific biomarkers for fluid-based detection of biliary tract cancer, as well as other diseases of the biliary tree. Metabolic profiling of bile using *in vitro* MRS is a valuable experimental tool for the identification of such early biomarkers of the disease.

### Bile physiology by MRS

The majority of MRS studies on bile have been carried out using $^{1}$H MR spectroscopy. The hydrophobic association of conjugated bile acids and biliary lipids in micelles has been confirmed in early MR studies[58]. Bile acids have also been quantified in both animal and human bile using *in vitro* $^{1}$H MRS. Rat bile studies with $^{1}$H MRS have derived peak assignments for C-18 methyl proton of bile acids at 0.7 ppm and the C-19 at 0.9 ppm, while the taurine moiety of taurine-conjugated bile acids resonated at 3.1 ppm and 3.5 ppm, respectively[59]. Conjugated bile acids in human gallbladder bile have been quantified using two dimensional MR[60]. Amide (-NH) proton resonances in glycine- and taurine-conjugated bile salts are in the region of 7.8-8.1 ppm[60].

More recently, $^{1}$H MRS has demonstrated cholesterol in human bile that can be differentiated from other lipid components in bile[61]. It has also been utilized for studying the effects of cholesterol on the fluidity of human gallbladder bile as well as for quantifying micellar PTC concentrations[61,62].

$^{31}$P NMR spectroscopy has been used to quantify phospholipids in red cell membranes and in model bile salts[63]. Such $^{31}$P MRS studies have shown higher concentrations of PTC and Pi in gallbladder bile compared to canalicular bile[64].

### MR spectroscopy of bile in disease

The biliary epithelium is exposed to numerous constituents of bile. Bile acids, PTC and cholesterol are of particular importance in disease if the cytoprotecive mechanism of cholangiocytes is disrupted[65]. Biliary disease has a major effect on biliary composition as the level of cellular function determines the production and biochemical constituents of bile. Biliary composition of these lipids also varies in cholestatic diseases of the liver and malignancy of the biliary tree. Current applications of

MRS in biliary disease include assessment of bile content in cholesterol gallstones, rejection in liver transplantation, primary biliary cirrhosis and biliary tract malignancy, as well in biliary excretion of xenobiotics[64,66-69].

MRS has been used to assess the distribution of biliary lipids between vesicles and micelles, which is believed to have a role in gallstone disease, and there is evidence that cholesterol from vesicular aggregates may be responsible for the deposition of cholesterol stones in the gallbladder[70]. Changes in the pattern of fatty acids of PTC with an increase of arachidonic acid have also been observed in bile from patients with gallstones[71]. Fusion and aggregation of phospholipid-cholesetrol vesicles which form liquid crystalline droplets leading to nucleation of cholesterol monohydrate crystals are thought to be responsible for the formation of cholesterol gallstones[70]. A selective reduction in biliary phospholipids has been suggested to be responsible for cholesterol gallstones in certain populations[72].

Initial $^{31}$P MRS studies on bile from patients with primary biliary cirrhosis have shown reduced levels of PTC and Pi when compared to bile from healthy volunteers[64], but the significance of these findings still remains to be determined in more extensive studies.

*In vitro* MRS has also been demonstrated to effectively identify and quantify xenobiotic metabolites in human and animal bile. Dioxins have been identified in biliary-cannulated rodents[67].

*In vitro* $^{1}$H MRS analysis of bile has also been applied in human liver transplantation in an attempt to assess donor liver integrity. Melendez and colleagues studied twenty-four hepatic bile samples from eight liver donors[69]. The livers from two donors were steatotic on histological analysis, while the rest were normal. $^{1}$H MRS analysis could show more intense PTC resonances in bile from steatotic donor livers, compared to bile from histologically normal donor livers. Seventeen hepatic bile samples from four recipients collected immediately after donor liver reperfusion were also analyzed by $^{1}$H MRS and showed bile from donor livers with good early graft function had a progressive increase in the bile acid peaks which represents restoration of bile flow. When compared to grafts with early graft dysfunction, the relatively reduced bile acid peaks in these spectra suggested slow recovery of bile secretion. In this study a total quantification of bile acids was not feasible due overlapping signals from other biliary lipids, notably cholesterol and PTC[69].

Our preliminary studies have revealed differences in phospholipid metabolites that may help distinguish between malignant and non-malignant causes of pancreaticobiliary obstruction. A reduced PTC resonance was seen in the bile from the majority of patients with hepatobiliary cancer compared to bile from patients with non-malignant indications for ERCP. This preliminary observation was confirmed by significant differences in the peak area ratios of PTC, referenced to the TSP standard in the $^{1}$H MR spectra ($P = 0.007$)[68].

Nishijima and colleagues have observed a lactate peak in bile spectra from patients with hepatic and biliary malignancy but not in bile from patients with non-malignant disease or bile from healthy controls[73]. The significance of this finding has yet to be determined in larger studies, where the collection and storage of bile for analysis are performed according to uniform protocols without potential contamination from ERCP contrast agents or the uncertainty of a long storage time that may lead to lactate accumulation.

## FUTURE STUDIES

Although MRS is primarily a research tool and its use to study hepatobiliary disease is a relatively new area, the ability of both *in vivo* and *in vitro* MRS to provide quick, repetitive, and non-invasive assessments of organ function raises several possible future development areas, including non-invasive diagnosis and staging of disease.

*In vitro* MRS holds a particular promise in the metabolic profiling of body fluids, such as urine, plasma and bile to pinpoint the potential disease mechanisms and to assess the response of the body to treatment regimens. This is of particular relevance when patients are having *in vivo* MRS studies, since an *in vivo*/*in vitro* correlation of the metabolite profile obtained may highlight disease processes and inform further genetic profiling studies[39].

Several clinical areas may be of potential interest. In the oncology field, phospholipid profiling to assess changes by *in vivo* $^{31}$P MRS may be useful in monitoring responses to the treatment of hepatic tumors, with co-temporaneous *in vitro* MRS analysis of plasma and urine. For example, chemoembolization of hepatic tumors has been found to correlate with a decrease in PME and increase in ATP concentrations using *in vivo* hepatic MRS[74].

Correlative *in vitro* MRS of body fluids and *in vivo* hepatic MRS techniques can also be applied to chronic liver disease to monitor liver fibrosis and progression towards cirrhosis. With increasing prevalence of alcohol-related liver disease, obesity-related liver disease (non-alcoholic hepatitis or NASH) and viral hepatitis, such as hepatitis C in particular, a reliable and repeatable monitoring system is required that obviates the morbidity and mortality associated with liver biopsy. *In vivo* and *in vitro* MRS may hold some of the answers to this, but further, larger scale studies are required to assess the true utility of these techniques with respect to other methodologies, such as microbubble contrast-enhanced ultrasound, ultrasound elastography and use of serological markers of fibrosis[75,76].

Future technical advances will boost the clinical potential of *in vivo* MRS, for example improving the delineation of multi-component signals, such as PME and PDE, with proton decoupling[77]. Increasing magnetic field strengths for *in vivo* studies may also provide better signal-to-noise and increased resolution, and 3T magnets are becoming steadily more available. Furthermore, *in vivo* $^{1}$H MRS may find a role in monitoring hepatic lipid content in interventional treatment studies of patients with non-alcoholic fatty liver disease in the future. *In vivo* $^{13}$C MRS for measurement of lipid metabolism, glycogen storage and gluconeogenesis looks promising for the future, but current sensitivity issues mean that this capability is currently confined only to a few centers[78-80]. *In vitro* MRS will find a correlative role in this context for screening plasma and urinary metabolites.

With respect to *in vitro* applications in bile, the ratio of taurine to glycine conjugates of bile acids and conjugated to unconjugated bile acids varies in hepatobiliary disease, and it has been widely accepted that elevated levels of bile acids in hepatocytes are toxic, leading to cholestasis[65]. More recently, the role of bile acids in cholangiocytes has been highlighted. *In vitro* studies on bile acids has demonstrated that they can act as ligands for the epidermal growth factor receptor on cholangiocytes and *via* the mitogen-activated protein kinase cell signalling pathway, leading to disordered cell cycling and cholangiocyte proliferation[81]. The measurement of bile acids is therefore necessary for an understanding of the pathophysiology of these diseases. *In vitro* MRS may find a useful role in screening bile in this context.

It is true that the significance of *in vivo* MRS needs to be assessed in larger studies with greater numbers of patients with various hepatobiliary diseases. Correlative *in vitro* NMR spectroscopy will help to elucidate some of the conundra. Trials in large populations in well-defined clinical settings are needed to determine if both *in vivo* and *in vitro* MRS can provide independent diagnostic and prognostic indices in management.

## ACKNOWLEDGMENTS

Some of the studies outlined were funded by the British Medical Research Council (MRC grant G99001978). Philips Medical Systems (Cleveland, Ohio, USA) are thanked for support of the magnet systems at the Robert Steiner MRI Unit, Hammersmith Hospital, Imperial College, London. We thank Adrian Lim, Shahid Khan and Nayna Patel for useful discussions.

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**S- Editor** Wang J **L- Editor** Wang XL **E- Editor** Bi L

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*World J Gastroenterol* 2006 August 14; 12(30): 4784-4787
***World Journal of Gastroenterology*** ISSN 1007-9327


*EDITORIAL*

# Contribution of genetics to a new vision in the understanding of inflammatory bowel disease

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Received: 2006-03-25 Accepted: 2006-04-24

## Abstract

Inflammatory bowel diseases (IBD), such as Crohn's disease (CD) and ulcerative colitis (UC), are chronic inflammatory autoimmune conditions of the gastrointestinal tract. Other organs, such as the eyes, skin and articulations, are often affected and IBD may be accompanied by other diseases of autoimmune origin. There is no single etiological factor responsible for the onset of IBD. Recent advances in genetics and in the molecular mechanisms of the proteins coded by these genes have given rise to a new vision in understanding these complex diseases. Activation of specific genes that affect antigen presentation and the handling of cells by innate immunity may lead to autoimmunity with the consequent activation of the major histocompatibility complex (MHC) and multiple cytokines involved in the regulation of acquired immunity. In this review IBD is described as a constellation of diseases that can best be classified as barrier diseases. This vision, developed by Kiel in Germany, includes the idea that changes in our environment due to the westernization of civilization have not been met with adaptation of the innate immune system, and this has given rise to autoimmune diseases. These diseases affect 1-5 of 1000 individuals and represent a major burden on the national health systems of many countries on different continents. On a world scale, a major challenge is to generate interventions to prevent the development of these diseases in Asia, Latin America and Africa.



**Key words:** Inflammatory bowel disease; Crohn's disease; Ulcerative colitis; Genetics; Autoimmunity; Major histocompatibility complex

Peña AS. Contribution of genetics to a new vision in the understanding of inflammatory bowel disease. *World J Gastroenterol* 2006; 12(30): 4784-4787

http://www.wjgnet.com/1007-9327/12/4784.asp

## INTRODUCTION

In less than five years after the discovery of the first gene involved in susceptibility to Crohn's disease (CD), the initial concept that CD is a multifactorial and polygenic disease has been consolidated [1,2].

Although the detailed functions of the *NOD2* or *CARD15* gene of chromosome 16 is not entirely clear, their relationship to intestinal flora, Toll receptors (TLR), and other intra-cytoplasmatic receptors of the NOD family together with their relation with NF-κB, has made clear that the innate immune response is of paramount importance in the pathogenesis of CD[3-5].

Two years ago it was suggested that the relationship between *TLR-2* and *NOD2* genes could explain the balance between activation of superficial receptors of the epithelial and dendritic cells to stimulate the production of NF-κB and NOD2 as inhibitors in order to prevent chronic disease[6-8]. According to this concept, individuals with mutations in the *CARD15* gene are not able to control the intestinal inflammation and this induces a TH1 immune response[9]. Recently, however, mutations in the terminal N of the gene in a region rich with leucine repeats have been transfected into the NOD2 region of mice[10]. Surprisingly, when these mice are challenged with muramil dipeptide (MDP) they generate considerable production of NF-κB and IL-1B[10]. Also the transfection of these mutations in HEK293 cells results in increased transcription of TNF-α, which suggests that different genes contribute to susceptibility to the disease and differences in manifestations of CD[11].

The fact that NOD2 is preferentially expressed in Paneth cells in the ileocaecal region probably explains the strong association between these mutations and this disease localization[12,13]. This region is rich in defensins, which are natural antibiotics that contribute to the mucosal barrier and innate immunity[14]. It is also now known that patients with mutations in the *CARD15* gene also have less α-defensins. This defect probably contributes to the role of intestinal flora in inducing and/or maintaining inflammation[15]. Preliminary results of a study carried out by the Stange group in Germany suggest that defensin deficiency is genetically determined. The defensin family is more complex and richer than originally described and its distribution varies within the gastrointestinal tract. The β-defensins are localized in the colon and defective β-defensins could contribute to the colonic localization of IBD[16]. It is also possible that different defensins may be specific to CD and UC variations[17].

These observations demonstrate the fine-tuning of

molecular biological responses of the gastrointestinal tract and the complexity of interactions among different genes on different chromosomes. Another example of complexity is gene polymorphisms of the gene inhibitor of plasminogen (PAI-1) in combination with *CARD15* mutations have an influence in the development of CD. Those patients who have mutations of *CARD15* and carry the *PAI-14G/4G* genotype develop a stricturizing phenotype (OR, 4.64; 95% CI, 1.26-17.05)[18].

Despite the well-demonstrated and replicated role of *CARD15* mutations in the susceptibility for CD in the majority of Caucasian populations[19-23], no mutations have been found in Asian[24-27] and in several European populations. In Scotland, Ireland, Galicia, Sweden and Finland the carriership of mutations in the *CARD15* gene is less frequent[28-32]. An interesting phenomenon was recently reported in monozygotic twins. In Sweden, the number of carriers of these mutations was as low as in the general population[32]. However, in Denmark 40% of the monozygotic twins carried *CARD15* mutations, which was a higher rate than in the Danish population with CD[33]. This corroborates the observations in Finland where the 1007fs allele frequency was higher in familial CD than in non-familial cases with CD (10.9% *vs* 3.5%; $P < 0.01$)[30]. These observations underscore the incidence of genetic variability and the importance of studying healthy controls in the general population.

## RELEVANCE OF OTHER GENES

Several other genes on other chromosomes are involved in determining susceptibility to CD. Two interesting genes, one on chromosome 5 and one on chromosome 10, contribute to the new vision of the genetics of CD. On chromosome 5, the SLC22A4/SLC22A5 haplotype codes for molecules involved in cationic transport, other solutes and carnitine (OCT-1 y OCT2)[34-36]. In some populations, an epistatic interaction has been found to exist between the *CARD15* mutations and the 250 KB region of 5q31[37,38]. The other interesting gene in this context is the DLG5 on chromosome 10, which is a gene that is important in the scaffolding of the epithelial cell[39,40].

The 250 KB region of 5q31 and the HLA region of 6p21 contain several genes that are of paramount importance in the regulation of the immune response and probably contribute to the phenotype of the patient with IBD. The *HLA-DRB1*0103* allele is associated with UC and with the colonic localization of CD[41,42].

Carriers of *HLA-DRB1*0103*, *B*35* and *B*27* have higher risk for arthralgias/arthritis in some of the greater articulations and *HLA-B*44* carriers are at higher risk for symmetric poly-arthritis[43,44].

A meta-analysis of 1068 CD patients has also implicated the genes described above in the development of CD and this study identified other regions of potential relevance on chromosomes 2q, 3q, 17q and 19q[45].

## THE CONTRIBUTION OF GENETICS TO A NEW VISION

In the new vision of CD, several genes are involved in the maintenance of the intestinal barrier, such as scaffolding genes (*DLG5*[46]), genes involved in the transport of key molecules for the homeostasis or exclusion of toxins (*SLC*[47] and *MDR1*[48,49]), genes involving the sensing of bacteria, both on the surface (TLR, CD14) and intracytoplamatically (CARD15, CARD4 and CARD8[50-52]. Whether a specific gene for regulating permeability exists, as has been suggested on chromosome 19, remains to be demonstrated. The combination of gene rich regions of chromosome 5 and 6 involved in regulating the immune response may contribute to the phenotype of the disease.

In summary, in less than a decade the genetics of IBD has evolved from epidemiology to molecular biology, and from observational studies to functional studies. The challenge for the coming years is the discovery of gene-gene interactions and gene-environment interactions.

A delineation of phenotypes based on genetic and molecular mechanisms will improve diagnoses and more accurate prognoses. This knowledge, together with advances in the understanding of the phenomenon of tolerance and the disruption of this mechanism in the understanding of chronic inflammation of the gastrointestinal tract, will lead to the design of better and novel therapeutic strategies. This should be the basis for effective drug development in addition to increasing knowledge generated by pharmacogenetics and pharmacogenomics.

Regarding the role of the environment in those individuals with a genetic susceptibility, new findings on epigenetic effects from long-term follow-up in monozygotic twins will open a new area of investigation. Investigators at the Spanish National Cancer Centre (CNIO) in Madrid have shown that older monozygotic twins exhibited differences in their overall content and genomic distribution of 5-methylcytosine DNA and histone acetylation. These epigenetic changes affect their gene-expression patterns and may explain the well-known disease discordance in these cases of monozygotic twins[53].

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**S- Editor** Pan BR **L- Editor** Lutze M **E- Editor** Ma WH

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*World J Gastroenterol* 2006 August 14; 12(30): 4788-4793
***World Journal of Gastroenterology*** ISSN 1007-9327


*REVIEW*

# Host susceptibility to persistent hepatitis B virus infection

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Received: 2006-04-03 Accepted: 2006-04-24

## Abstract

Genetic epidemiology researches such as twin studies, family-clustering of hepatitis B virus (HBV) infection studies and ethnic difference studies have provided the evidence that host genetic factors play an important role in determining the outcome of HBV infection. The opening questions include which human genes are important in infection and how to find them. Though a number of studies have sought genetic associations between HBV infection/persistence and gene polymorphisms, the candidate gene-based approach is clearly inadequate to fully explain the genetic basis of the disease. With the advent of new genetic markers and automated genotyping, genetic mapping can be conducted extremely rapid. This approach has been successful in some infectious diseases. Linkage analysis can find host genes susceptible to HBV and is of great clinical importance.



**Key words**: Hepatitis B virus; Susceptibility; Association study; Linkage study

He YL, Zhao YR, Zhang SL, Lin SM. Host susceptibility to persistent hepatitis B virus infection. *World J Gastroenterol* 2006; 12(30): 4788-4793

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## INTRODUCTION

Hepatitis B virus (HBV) infection is a serious global health problem, with 2 billion people infected worldwide, and 350 million suffering from chronic HBV infection. HBV infection results in 500 000 to 1.2 million deaths per year caused by chronic hepatitis, cirrhosis, and hepatocellular carcinoma and is the 10th leading cause of death worldwide[1]. The mechanisms of persistent HBV infection are not fully understood, but they seem to involve several aspects and the genetic component, in particular, is still controversial[2]. Early studies by Blumberg *et al*[3] have also suggested a recessive mode of inheritance for HBV viral persistence, but this is perhaps an oversimplification giving the more recent advances in knowledge of the effect of maternal viral infection and the transmissibility of the virus[4].

Generally, exposure to HBV can cause a broad spectrum of infection[5]. Ninety to ninety-five percent of adults infected with HBV can eliminate the virus and only 5%-10% of them become chronic HBV carriers, 20%-30% of chronic HBV carriers develop chronic hepatitis B (CHB) and 5% of them develop liver cirrhosis and hepatocellular carcinoma in a long term of disease course. Some rare cases result in a fulminant infection in which the liver is rapidly overwhelmed and ultimately fails. What factors determine why one develops a life-threatening infection, whereas another carries HBV as a harmless commensal or limits the infection to a clinical trivial episode? There is evidence that host genetic factors play an important role in determining the outcome of HBV infection[6,7].

## EPIDEMIOLOGY EVIDENCE FOR HUMAN GENETIC SUSCEPTIBILITY TO PERSISTENT HBV INFECTION: TWINS, FAMILY-CLUSTERING OF HBV INFECTION AND ETHNIC DIFFERENCES

### *Twin studies*

Studies of susceptibility to diseases in identical and non-identical twins are extremely useful in evaluating the importance of inherited *vs* environmental factors in disease susceptibility[8]. If the concordance rates for infection and clearance of HBV are significantly higher in monozygotic (MZ) than in dizygotic (DZ) twins, the process of HBV infection and persistence is more genetically decisive. Lin *et al*[9] studied 289 pairs of MZ twins, 102 pairs of DZ twins and 375 pairs of age-sex-matched singleton controls and found that there is a significant difference in the concordance of HBV infection between MZ and DZ twins and controls, suggesting that the genetic influence occurs in response to HBV infection. Xu *et al*[10] also found that not only the concordance rate of infection, but the concordance of clinical phenotype and serological

patterns between MZ and control groups is significantly different, indicating that genetic factors influence not only susceptibility to infection but also clinical outcome.

Genetic factors not only influence host response to HBV infection, but also affect the response to HB vaccine. Hohler *et al*[11] prospectively studied and vaccinated 202 twin pairs with a combined recombinant HBsAg vaccine and found that the heritability of anti-HBs immune response is 0.61, which means that 60% of the phenotypic variance of responsiveness to HB vaccine can be explained by genetic effect and 40% by environmental effect.

### *Family-clustering of HBV infection*

Most of us do not inherit single-gene diseases. We all, however, inherit slightly different variants of each of our pairs of 30 000 genes. These differences may determine whether we are more or less likely to develop particular health problems or diseases than other people. Genes are shared within families. Because we inherit genes from our parents, a parent who has inherited a particular gene mutation generally means that each child has a fifty-fifty chance of having the same mutation. In fact, many cases of diseases not showing the clear inherited patterns of single-gene diseases, show family clustering patterns that are due, at least in part, to genetics. Substantial genetic epidemiology studies indicate that HBV spreads in families. The familial occurrence of HBV infection has been well established in some ethnic groups. Ohbayashi *et al*[12] have reported 3 Japanese families in which 36 of the 54 members are HBsAg positive. Of these, some are healthy carriers while others have liver cirrhosis and hepatocellular carcinoma. Similar observations have been reported in American[13], European[14] and Asian[15,16] continents.

This observed familial clustering may stem from inherited defects in specific genes, from shared environmental exposures among family members or from interaction between specific genetic and environmental factors. If a trait has a genetic basis, the relatives of affected individuals will be affected more frequently than the relatives of unaffected people, and the prevalence of disease decreases from monozygotic (MZ) twin to the first-, second- and third-degree relatives. If the disorder has an environmental basis only, the possibility of infection in each family member is equal[17]. Tong *et al*[18] reported that HBV markers are detected more frequently in blood relatives than in non-blood relatives of the index cases in family. Wang *et al*[19] also showed that HBsAg carrier rate decreases in the order of the first, second and third degree relatives, indicating that it is the defect gene shared by family members that produces the epidemiological characteristics of family-clustering HBV infection.

### *Ethnic differences*

Another method used to investigate the role of host genetics in infectious diseases is to look for differences in clinical disease and immune response between different ethnic groups having equal exposure to the same pathogen. Carrilho *et al*[20] determined the frequency of HBV markers of genetically related (consanguineous) and non-genetically related (non-consanguineous) Brazilian families of Asian origin and Western origin and found that the occurrence of HBsAg is significantly higher ($P < 0.0001$) in family members of Asian origin (81.8%) than in those of Western origin (36.5%), which is in line with the high HBsAg prevalence in Asian countries and the relatively low HBsAg prevalence in Western countries[21]. Though the Asians live in Brazilia, a country with a low HBsAg prevalence, and the environment has changed, disease-related genes remain shared within the ethnic group, indicating that Asians possess the HBV susceptible gene(s). This is why they are more susceptible to HBV.

Tong *et al*[18] tested family members of Asian and non-Asian patients for HBV markers, and found that Asian family members have a significant increase in HBsAg (34% higher) and antibodies to HBsAg or to hepatitis B core antigen (50% higher) compared with the non-Asian family members. Moreover, birthplace, either in Asia or in United States, does not influence the frequency of antigenemia. In China, the prevalence of HBsAg is 19.1% in Mongoloid populations[22], and 10% in Chinese Han populations in the same area. These studies have provided important insights into the fact that different ethnics in the same region have different HBV epidemiological characteristics and the same races in the different region share the same prevalence of HBV markers, indicating that genetic factors may play a role in maintaining the frequency of HBV infection and persistence. Moreover, molecular epidemiology study has identified several genetically determined differences between races.

Taken together these epidemiological data provide strong evidence for a genetic predisposition to HBV infection and raise the questions of which human genes are important in infection and how to find them.

## TWO METHODS USED TO IDENTIFY HBV SUSCEPTIBLE GENES

Analysis of the human genome has focused primarily on variations that occur between people in their DNA sequence[23]. Because these differences contribute to the differences in our susceptibility to developing specific diseases, naturally occurring genetic variations in the human genome are frequently found (about every 3 to 500 bp) most often in the form of a change from one base to another, namely a single nucleotide polymorphism (SNP)[24]. Other common forms of variation include microsatellite where a short sequence, usually a dinucleotide repeat is bound, so that one person might have 10 and 12 copies of the repeating motif and others have 9 and 11 copies. If the repeating sequence is longer, the motif is known as a minisatellite[25]. They are widely used to determine similarities and differences of human and hunter disease-related genes. Because this kind of genetic variations often varies between individuals (i.e., it is highly polymorphic), microsatellites are particularly informative in the genetic sense[26]. Analysis of genetic susceptibility to HBV infection aims to link these DNA variations (the genotype) with a particular HBV infection (the phenotype). HBV infection and clearance are complex traits[27], meaning that the genetic contribution to them is not inherited in a

**Table 1 Gene polymorphisms associated with clearance of HBV infection**

| Gene/loci | Population | Sample size | *P* value | Reference |
|---|---|---|---|---|
| HLA A 0301 | Caucasian | 563 | 0.0005 | [30] |
| HLA -DRB1 1302 | Caucasian | 563 | 0.03 | [30] |
| HLA-DRB1 1302 | Gambian | 638 | 0.012 | [31] |
| HLA-DRB1 1101/1104 | Chinese | 190 | 0.0145 | [32] |
| HLA-DQA1 0301 | Chinese | 190 | 0.0167 | [32] |
| HLA-DR13 | Korean | 1272 | < 0.001 | [33] |
| TNF-alpha-238 GG genotype | Chinese | 895 | 0.041 | [34] |
| TNF-alpha-308 A | Korean | 1400 | < 0.001 | [35] |
| TNF-alpha-857 TT genotype | Chinese | 355 | 0.02 | [36] |
| CTLA-4-1722 C | | | 0.06 | [37] |
| CTLA-4+49 G | | | 0.02 | [37] |
| CCR5 59029 G allele | Chinese | 377 | 0.001 | [38] |

**Table 2 Gene polymorphisms associated with susceptibility to chronic hepatitis B**

| Gene/loci | Population | Sample size | *P* value | Reference |
|---|---|---|---|---|
| HLA B 08 | Caucasian | 563 | 0.03 | [30] |
| HLA B 44-Cw 1601 | Caucasian | 563 | 0.02 | [30] |
| HLA B 44-Cw 0501 | Caucasian | 563 | 0.006 | [30] |
| HLA-DRB1 0301 | Chinese | 190 | 0.0074 | [32] |
| HLA-DRB1 1301/2 | | | | [39] |
| HLA-DR6 | Korean | 1272 | < 0.001 | [33] |
| HLA-DQA1 0501 | Chinese | 190 | 0.0157 | [32] |
| HLA -DQA1 0501 | African American | 91 | 0.05 | [40] |
| HLA -DQB1 0301 | African American | 91 | 0.01 | [40] |
| HLA-DQB1 0301 | Chinese | 190 | 0.0075 | [32] |
| TNF-alpha-863 A | Korean | 1400 | | [35] |
| TNF-alpha-238 GG genotype | Chinese | 355 | 0.02 | [36] |
| TNF-alpha-238 GG genotype | Chinese | 455 | 0.02 | [41] |
| TNF-alpha-857 CC genotype | Chinese | 895 | < 0.001 | [34] |
| IFN-gamma A/A genotype | | 77 | | [42] |
| CTLA-4+6230 A | | | 0.04 | [37] |
| CCR5 59029 A allelic genotype | Chinese | 377 | 0.002 | [38] |
| ESR1 29 T/T genotype | Chinese | 2318 | < 0.001 | [43] |

simple Mendelian manner and several polymorphic genes exert effects on the outcome[28]. Many possible approaches to mapping the genes underlying complex traits fall broadly into two categories: candidate gene- based association studies and genome-wide linkage studies[29].

### Association studies

Association studies compare the frequency of alleles or genotypes of a particular variant between disease cases and controls to link the genotype with the particular phenotype. Such studies are widely used to investigate inflammatory and infectious diseases. Repeat sequences, such as those of microsatellites, lend themselves less well to association studies because they are intrinsically unstable and may undergo considerable mutations over successive generations and disease-modifying polymorphisms may have arisen many hundreds of generations previously. SNPs, on the other hand, are stable, common and increasingly amenable to high throughput automated genotyping. A number of studies have sought genetic associations between HBV infection/persistence and gene polymorphisms (Tables 1 and 2).

The huge variation in clinical response to identical infecting pathogens is due to the combined effects of genetic variation both in the infecting pathogen and in the infected host[44]. Its ability to mount an effective immune response to infection is a powerful evolutionary selection pressure, contributing to human genetic diversity. The advantage of a flexible immune response, allowing an efficient response to diverse pathogens without damage to the host, is reflected in marked genetic variability of immune-related genes among (both in DNA sequence and in protein structure) in the entire human genome[45].

The prototype region for genetic association studies is the human leukocyte antigen (HLA) loci involved in antigen processing and presentation. HLA associated with infections such as AIDS[46], tuberculosis[47], leprosy[48], malaria[49] and persistence of hepatitis-C virus[50] has been well-described. This is most obvious within the HLA region, where functional variation has arisen as a strategy to combat pathogen antigenic diversity. Indeed in HBV infection, maximal HLA variation appears to have a direct protective effect, individuals with the most different alleles at class Ⅱ HLA loci have the slowest HBV disease progression and the lowest mortality (a "heterozygous advantage")[51]. Conversely, lack of HLA diversity (a "homozygous disadvantage") may increase the susceptibility to HBV infection among isolated communities[52]. The extensive linkage disequilibrium across some HLA regions makes it difficult to localize specific disease-associated polymorphisms, although the HLA allelic association has allowed identification of critically pathogenic epitopes in some diseases[39], which might act as potential vaccine candidates.

Disease associations involving loci outside the HLA region are also valuable in identifying the functional molecular basis underlying infectious disease resistance. For example, HIV uses various chemokine receptors as cofactors for CD4 binding to gain entry into human leukocytes. A functional polymorphism of the chemokine receptor CCR5, which is essential for HIV entry into macrophages, results in a truncated nonfunctional protein that confers highly significant protection against HIV susceptibility in the homozygous state and slows disease progression in heterozygotes[53,54]. Chang *et al*[38] have developed the association between CCR5 and HBV infection, though the biological process and significance in HBV infection need to be further studied.

### Shortcoming of association studies in susceptible gene hunting

The number of studies seeking to identify genes that influence susceptibility to persistent HBV infection has greatly increased since we entered the "post-genomic" era. These studies are fuelled by the unlimited availability of

**Table 3 Successful linkage analysis in infectious diseases**

| Diseases | Location of predisposing genes | Reference |
|---|---|---|
| *H pylori* | IFNGR1 | [60] |
| Plasmodium falciparum | 5q31-q33, MHC | [61-64] |
| Kala-azar | 22q12, Imr2, Imr1 | [65] |
| Tuberculosis | 15q and Xq | [66] |
| Schistosoma mansoni | 5q31-q33 | [67] |
| Leprosy | 10p13, 6q25 | [68, 69] |

SNPs, the relative ease of performing genotyping assays based on PCR technology, and the desire to identify major disease susceptibility gene(s). Literature is now littered with unreproducible genetic association studies that confuse the readers and have an understandable impact on the willingness of editors to accept further manuscripts for publication[27]. *Nature Genetics* published an editorial in 1999 that set out a list of criteria for genetic association studies[55]: plausible biological context, rigorous phenotypic selection (case selection), independent replication, rigorous genotyping, low *P* values, appropriate statistical analysis, and transmission disequilibrium test. Up to now, few candidate genes can fully meet the criteria.

Candidate gene-based association studies rely on having predicted the identity of the correct gene or genes, usually on the basis of biological hypotheses or the location of the candidate within a previously determined region of linkage[56]. Even if these hypotheses are broad (for example, involving the testing of all genes in the insulin-signaling pathway), they will, at best, identify only a fraction of genetic risk factors for diseases in which the pathophysiology is relatively well understood. When the fundamental physiological defects of a disease are unknown, the candidate-gene approach is clearly inadequate to fully explain the genetic basis of the disease[29]. In 2004, *Hepatology* editor appealed for less hypothesis-driven association studies that result in a negative or weak correlation[27].

### *Linkage studies*

Linkage is the tendency for genes and other genetic markers to be inherited together because of their location near one another on the same chromosome. Linkage studies classically seek to identify microsatellite markers that are inherited more commonly than expected by siblings who have the disease of interest ("affected sibling pairs")[57]. Genetic linkage analysis is a powerful tool to detect the chromosomal location of disease genes[58]. A linkage study is to use a large number of families to look for regions of linkage to a disease, which suggest the presence of loci containing genes that may predispose to this disease. Linkage studies have the advantage of making no supposition about which genes might be involved in a disease, in that they merely identify stretches of chromosome around the microsatellite markers and can be used to examine the entire genome (a "whole genome screen")[58].

With the advent of new genetic markers and automated genotyping, genetic mapping can be conducted extremely rapid[28,59]. This approach has been successful in some infectious diseases (Table 3), but no report on such similar scans for HBV viral persistence is available. Recently a research team of Xi'an Jiaotong University has collected 327 HBV-infected subjects of 32 family pedigrees from a remote village (data not published), which makes it possible to find chromosome regions containing determinant(s) of persistent HBV infection. Their results will be reported soon.

## CLINICAL IMPLICATION OF GENETIC STUDIES OF HBV INFECTION

Studies of the genetic determinants for HBV susceptibility can reveal fundamental data concerning the human immune system. The ultimate goal of such studies is the identification of critical immunologic mechanisms in the disease process to develop specific therapeutic interventions. As the precise immune deficiency is identified, it may be possible to "bypass" the identified immune deficiency with a specific therapy.

A specific genetic defect has been identified in rarer single gene defects, which may offer preconception genetic counseling to affected families. In complex diseases it might ultimately be possible to identify patients whose risk factors make them candidates for targeted therapies. Once the genotypic markers for a poor outcome of HBV infections are found, they in combination with rapid genotyping technology may allow more intensive therapies for those patients who are at the greatest risk of poor outcome and death[70,71]. The potential to target drug treatment, both in terms of identifying patients most likely to benefit clinically and in terms of predicting those who are susceptible to either favorable or adverse pharmacologic outcome, is of great importance. It is conceivable that in the future our understanding of host genetics will largely influence our therapeutic response to HBV-infected patients and determine our choice of both preventive and curative interventions.

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**S- Editor** Wang J **L- Editor** Wang XL **E- Editor** Bai SH

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*World J Gastroenterol* 2006 August 14; 12(30): 4794-4806
***World Journal of Gastroenterology*** ISSN 1007-9327


*TOPIC HIGHLIGHT*

**Paolo Gionchetti, MD,** *Series Editor*

# Conventional therapy for Crohn's disease

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Received: 2006-02-17 Accepted: 2006-03-10

## Abstract

Crohn's disease (CD) is a multifactorial disorder of unknown cause. Outstanding progress regarding the pathophysiology of CD has led to the development of innovative therapeutic concepts. Numerous controlled trials have been performed in CD over the last years. However, many drugs have not been approved by regulatory authorities due to lack of efficacy or severe side effects. Therefore, well-known drugs, including 5-ASA, systemic or topical corticosteroids, and immunosuppressants such as azathioprine, are still the mainstay of CD therapy. Importantly, biologicals such as infliximab have shown to be efficacious in problematic settings such as fistulizing or steroid-dependent CD. This review is intended to give practical guidelines to clinicians for the conventional treatment of CD. We concentrated on the results of randomized, placebo-controlled trials and meta-analyses, when available, that provide the highest degree of evidence. We provide evidence-based treatment algorithms whenever possible. However, many clinical situations have not been answered by controlled clinical trials and it is important to fill these gaps through expert opinions. We hope that this review offers a useful tool for clinicians in the challenging treatment of CD.



**Key words:** Crohn's disease; Conventional treatment; Review; Therapy

Büning C, Lochs H. Conventional therapy for Crohn's disease. *World J Gastroenterol* 2006; 12(30): 4794-4806

http://www.wjgnet.com/1007-9327/12/4794.asp

## INTRODUCTION

Crohn's disease (CD) is a chronic bowel disease characterised by a relapsing inflammatory process. It can affect any part of the gastrointestinal tract and is associated with discontinuous, transmural lesions of the gut wall. The current working hypothesis suggests that CD results from an aberrant immune response towards fecal bacteria in a genetically susceptible host[1].

While medical treatment of the acute flare is successful in most patients, one of the most difficult tasks in general medicine is to treat complications such as strictures, abscesses, fistulae and chronic disease activity. In this review, we describe the conventional treatment of CD depending on different clinical situations, such as an acute flare, maintenance of remission, fistulizing or chronically active disease behaviour.

Apart from the below discussed medical and surgical treatment of CD, other factors including changes in lifestyle should be recommended. Herein, probably the most important aspect is smoking cessation. Smoking has shown to be a risk factor for CD relapse after medically or surgically induced remission[2] and is associated with the need for higher doses of corticosteroids and immunosuppressants[3]. Importantly, a prospective trial showed that only one year of smoking cessation leads to a more benign course of disease with a lower rate of relapses[4]. This trial also showed that the ability to quit smoking clearly depended on the physician's role. So the conventional treatment of CD should start, if neccessary, with convincing the patient to quit smoking.

## ACTIVE DISEASE

### *Definition*

The activity of CD can be assessed clinically, endoscopically or by other indices[5]. The most established way is through the BEST activity index (CDAI), where symptoms and objective criteria such as anemia and body weight are included[6]. Index values of 150 and below are associated with quiescent disease; values above that indicate active disease, and values above 450 are seen with extremely severe disease. In addition other diagnostic values such as blood sedimentation rate, C-reactive protein (CRP) and thrombocytes should also be taken into account. Endoscopic inflammatory evaluation, however, is not necessary in every exacerbation of the disease but might offer important information with respect to disease localisation. This is especially important for the use of topically acting agents such as budesonide in terminal ileal or right colonic CD. Exacerbation of CD through infectious agents should always be considered and

**Table 1 Drugs for the treatment of CD**

| Drug | 5-ASA (mesalamine or sulfasalazine) |
|---|---|
| Dosage | 3.2-4 g/d |
| Indications | Mild to moderately active disease, postoperative maintenance |
| Important side effects | Headache, nausea and abdominal pain, often during treatment with sulfasalazine (in up to 45% of patients); thrombopenia; interstitial nephitis, pancreatitis; |
| Monitoring | Liver function, full blood count and especially renal function |
| Pregnancy | Suggested to be safe in conventional doses |

excluded if possible.

In addition, the American College of Gastroenterology has defined the different disease activities in clinical practise as follows[7]: mild to moderately active disease is defined as "ambulatory patients able to tolerate oral alimentation without manifestation of dehydration, toxicity (high fevers, rigors, prostration), abdominal tenderness, painful mass, obstruction or > 10% weight loss. In contrast, moderate to severe disease applies to patients that have failed to respond to treatment for mild to moderate disease or those with more prominent symptoms such as fever, significant weight loss, abdominal pain or tenderness, intermittent nausea or vomiting (without obstructive findings), or significant anemia. Severe disease refers to patients with persisting symptoms despite the introduction of steroids as outpatients, or individuals presenting with high fever, persistent vomiting, evidence of intestinal obstruction, rebound tenderness, cachexia, or evidence of an abscess.

### 5-ASA

No debate has been as longstanding and controversial as whether the use of 5-ASA containing drugs in CD is justified or not. Numerous studies regarding this aspect have been performed over the last 25 years. However, data from current studies do not clearly support either point of view. Different study designs and drug dosages have been used that make comparison of the results rather difficult.

Sulfasalazine is the original compound in this class consisting of 5-ASA linked by an azo-bond to sulfapyridine, which is split off in the colon. Therefore efficacy of sulfasalazine was expected to be limited to colonic disease. Furthermore up to 50% of patients are not able to tolerate sulfasalazine due to nausea, headache, vomiting and epigastric pain. These side effects are suggested to be caused by the sulfapyridine moiety. Therefore, other 5-ASA formulations (mesalamine formulations and the pro-drugs olsalazine and balsalazide) without sulfapyridine have been introduced into the market with different pharmacodynamic and pharmacokinetic profiles (Table 1). These different preparations are therefore suggested to be non-interchangeable.

**Sulfasalazine:** Sulfasalzine has been shown to be significantly better than placebo in randomized clinical trials in inducing remission in active CD[8-10]. Subgroup analyses suggested that patients with only colonic disease seem to benefit the most from sulfasalazine therapy[8,9], whereas patients treated previously with prednisone failed to respond[8]. Sulfasalazine has not shown to have steroid-sparing properties[9,11]. Since 5-ASA was identified to be the active moiety in sulfasalazine, other 5-ASA containing formulations (such as mesalamine) have been tested in CD.

**Mesalamine:** Different pharmacological preparations allow relase of the active drug in different parts of the intestine. Therefore mesalamine, in contrast to sulfasalazine, may also be used in CD including small bowel CD. However, studies on the induction of remission in active CD with mesalamine yielded conflicting results. In total, six placebo-controlled trials with varying dosages of mesalamine have been performed to date. Two earlier studies did not detect a benefit of mesalamine over placebo in inducing remission[12,13]. Tremaine and colleagues observed a significantly greater number of patients that responded (defined as either a decrease of CDAI $\geqslant$ 70 or CDAI < 150), but this benefit was rather small (9 patients with mesalamine treatment *vs* 4 patients in the placebo group). However, no significant differences were found when clinical remission (defined as CDAI < 150) was analyzed[14]. Singleton and colleagues conducted three different trials with mesalamine (Pentasa) that were recently combined in a meta-analysis although two of the three trials were never published in full[15]. This analysis found a statistically significant benefit of mesalamine over placebo. However, this benefit was rather small (CDAI reduction of 18 points for the intention-to-treat-analysis).

In summary the clinical benefit of mesalamine in the treatment of active CD seems to be rather low. However, mesalamine is well tolerated and has a favourable side effect profile compared to sulfasalazine. The latter factor is probably the main reason why mesalamine is significantly used more often compared to sulfasalazine although data from randomized trials are in favor of sulfasalazine. Furthermore, many patients with mild to moderately active disease try a more harmless drug at first before taking corticosteroids.

### Budesonide

The introduction of the topically-acting steroid budesonide has become a very potent alternative in the treatment of patients with CD located in the terminal ileum or right colon. Due to rapid metabolism by cytochrome P-450 enzymes in the liver, budesonide has less sytemic bioavailibility than systemic corticosteroids. A recent meta-analysis combined the data from 5 published studies investigating budesonide in comparison to placebo, 5-ASA and systemic corticosteroids[16]. A significant advantage of budesonide in inducing remission was observed in comparison to placebo (odds ratio of 1.85) and mesalamine (odds ratio of 1.73). Accordingly, a patient is 73% more likely to achieve remission with budesonide than mesalamine. Corticosteroids induced remission even more often as compared to budesonide with an odds ratio of 0.87, but in patients with mild and moderate disease (CDAI 200-300), no difference in remission rates was found. Treatment with budesonide was associated with similar side effects compared to mesalamine and placebo. Importantly, fewer side effects, such as acne, moon face

**Table 2 Drugs for the treatment of CD**

| Drug | Systemicorticosteroids (prednisone equivalent) or budesonide |
|---|---|
| Dosage | Corticosteroids: 30-60 mg/d or 1-1.5 mg/kg per day; Budesonide: 9 mg |
| Indications | Corticosteroids: moderate to severe disease. Budesonide: terminal ileal and right colonic disease in mild to moderate disease, low dose budesonide eventually for maintenace therapy |
| Important side effects | Weight gain, hypertension, fluid retention, myopathy, mood changes, infections, glaucoma, skin changes including acne, adrenal suppresion. Long term side effects: osteoporosis, cataract, aseptic bone necrosis |
| Pregnancy | Lower doses seem to be relatively safe |
| Comments | Avoid long-term use |

**Table 3 Drugs for the treatment of CD**

| Drug | Azathioprine (6-mercaptopurine) |
|---|---|
| Dosage | 2-2.5 mg/kg (1-1.5 mg/kg) |
| Indications | Maintenance, chronically active disease, steroid-refractory and steroid-dependency, fistulae, concomittant therapy with infliximab; |
| Important side effects | Pancreatitis, bone marrow supression, allergic reactions, drug heptatitis, nausea, malaise, bacterial and viral infections; in patients intolerant to azathioprine due to gastrointestinal symptoms, 6-mercaptopurine is suggested (not in side effects such as pancreatitis and bone marrow suppression) |
| Monitoring | Liver function, lipase and full blood count biweekly for the first three months, if normal then every three months throughout therapy |
| Pregnancy | Should be avoided, although available studies suggest a potential use especially in patients where maintaining remission is essential |
| Comments | Entire therapeutic efficacy is observed mostly after 2-4 mo; consider testing for thiopurine methyltransferase (TPMT) genotypes to identify patients with high-risk of bone marrow suppression; consider metabolite monitoring for adaequate dosing; ensure adequate birth control; allow 3 mo time before pregnancy or conceiving |

and osteoporosis, were observed compared to systemic corticosteroids. The recommended dose of budesonide is 9 mg/d and should be tapered 3 mg every 2-4 wk unless a maintenance therapy with budesonide is suggested (see below).

### Systemic corticosteroids

For moderate to severe CD, and especially if therapy with 5-ASA has failed, systemic corticosteroids are the treatment of choice (Table 2). Corticosteroids are fast and effective and induce remission in approximately 70% of patients. In active CD, corticosteroids have been shown to be superior to sulfasalazine, azathioprine and placebo[8,9]. No dose finding studies have yet been performed. Reported doses range from 30 mg/d to 1 mg/kg per day, however most clinicians start with 60 mg/d, although it seems to be favourable to apply a body weight dependent dosage (1 mg/kg). Tapering should be performed according to improvement of clinical symptoms and is usually done in steps of 5-10 mg/wk. At lower dosages, tapering might be reduced to 2.5-5 mg/wk. Whether i.v. application has an advantage over oral in severe acute flares is not clear, although it is frequently used when oral treatment has not been effective.

**Table 4 Drugs for the treatment of CD**

| Drug | Methotrexate |
|---|---|
| Dosage | 25 mg/wk i.m., if remission is achieved reduce to 15 i.m. (or s.c.) |
| Indications | Maintenance, chronically active disease, steroid-refractory and steroid-dependency, fistulae |
| Important side effects | Nausea, abdominal pain, diarrhea, stomatitis; hepatitis, liver fibrosis; hypersensivity pneumonitis |
| Monitoring | Liver function and full blood count monthly for the first two months, if normal then every two months throughout therapy |
| Pregnancy | Strictly prohibited |
| Comments | Entire therapeutic efficacy is observed mostly after 2-4 mo; consider folic acid supplementation with 2.5-5 mg/d; ensure adequate birth control; allow 3 mo time before pregnancy or conceiving |

### Azathioprine/6-Mercaptopurine

The most common-ly used immunomodulators are the thiopurines, 6-mercaptopurine and its prodrug azathioprine (Table 3). Numerous clinical trials studied the efficacy of these immunomodulators in active CD. The most convincing data were obtained in the early trial by Present and colleagues where 67% *vs* 8% of the patients in the 6-mercaptopurine group *vs* placebo, respectively, achieved remission[17]. However, other trials did not observe a significant difference in the use of azathioprine compared to placebo[8,18]. Despite these conflicting data, a meta-analysis reported an odds ratio of 3.09 favoring azathioprine/6-mercaptopurine therapy over placebo[19] to induce remission. In addition, a recent Cochrane analysis reported an overall response in active CD of 54% *vs* 33% for azathioprine *vs* placebo, respectively[20].

Thiopurines are slow acting drugs and an effect can be observed after 2-3 mo. Thus thiopurines are less frequently used to induce remission in an acute exacerbation but rather to maintain remission. However, they have been shown to have steroid sparing properties[19,20] and furthermore the combination of prednisolone and azathioprine has shown to be superior over prednisolone monotherapy[21]. Therefore it is suggested to add azathioprine to corticosteroids in severe CD.

### Methotrexate

In the pivotal trial by Feagan and collea-gues, methotrexate given intramuscularly 25 mg once a week was more likely to induce remission compared to placebo (Table 4). In addition, steroid-sparing properties were noted[22]. However, side effects were more common with methotrexate therapy than with placebo. Other studies using low dose methotrexate did not show a significant benefit[23,24]. In addition, no benefit was obserevd when high intravenous methotrexate was compared to oral

**Table 5 Drugs for the treatment of CD**

| Drug | Metronidazole |
|---|---|
| Dosage | 10-20 mg/kg |
| Indications | Mild to moderately active disease; fistulae (usually prolonged treatment) |
| Important side effects | Nausea, metallic taste in the mouth, coating of the tongue, peripheral neuropathy |
| Monitoring | See side effects |
| Pregnancy | Long term treatment not yet evaluated, short term treatment appears to be safe |

**Table 6 Drugs for the treatment of CD**

| Drug | Ciprofloxacin |
|---|---|
| Dosage | 1-2 g/d |
| Indications | Mild to moderately active disease, fistulae |
| Important side effects | Taste disturbance, gastrointestinal events, tendopathies |
| Monitoring | Generelly well tolerated, see side effects |
| Pregnancy | Probably safe |

azathioprine[25]. Like azathioprine/6-mercaptopurine, intramuscular methotrexate is only rarely used to treat an acute exacerbation of CD but is used more frequently in chronic active CD[26]. Importantly, side effects with methotrexate, specifically liver dysfunction, are common and need to be monitored. In addition, methotrexate is contradicted during pregnancy and should be used very cautiously in women of child-bearing potential.

### Antibiotics

Although antibiotics are frequently used to treat CD, this practice is not supported by strong evidence from randomized trials. However, increasing knowledge of the impotance of mucosal bacteria for the pathogenesis of CD gives a good rationale for investigating antibiotic approaches[27]. In addition, distinguishing an acute flare from an infectious gastroenteritis/colitis can be difficult. Thus antibiotics provide a therapeutic alternative, which might benefit both an acute flare and a gastrointestinal infection. However, further studies are warranted to establish the role of antibiotics in the treatment of CD and at this time they cannot be recommended as standard therapy.

**Metronidazole:** Metronidazole (20 mg/kg per day) has been shown to be superior over placebo in reducing the CDAI but not with respect to the induction of remission[28] (Table 5). Furthermore, this benefit was only seen in patients with colonic or ileocolonic disease, whereas no benefit was found with disease location in the ileum. Similar findings were reported from another trial where few patients with colonic involvement showed an improvement[29]. Another study reported no benefit *vs* placebo[30]. Compared to sulfasalazine, a cross over study reported no difference in the first 4 mo. However, in the cross over design, patients switched to metronidazole showed an improvement of CDAI, whereas in the sulfasalazine group this was not the case[31,32].

**Table 7 Drugs for the treatment of CD**

| Drug | Infliximab |
|---|---|
| Dosage | 5 mg/kg per infusion; usually started at wk 0, 2, and 6 and then repeated every 8 wk if necessary |
| Indications | Chronically active disease, steroid-refractory and steroid-dependency, maintenance, fistulae |
| Important side effects | Nausea, headache, abdominal pain, infections, sepsis; infusions reactions (early or delayed), reactivation of tuberculosis |
| Monitoring | Vital signs around infusion |
| Pregnancy | Unknown |
| Comments | Exclude tuberculosis before infusions, consider concommittant use of imunosuppressants (azathioprine) to reduce antibody formation |

**Ciprofloxacin:** Ciprofloxacin is often used in a clinical routine, especially in combination with metronidazole (Table 6). Ciprofloxacin was significantly better compared to placebo in inducing remission in a smaller trial[33] and was shown to be similarly effective compared to mesalamine[34]. In contrast, corticosteroids resulted in higher rates of clinical remission compared to ciprofloxacin and metronidazole[35]. In patients with chronically active disease on budesonide, the addition of metronidazole and ciprofloxacin was not superior over budesonide monotherapy, although in patients with colonic CD a trend towards a significant benefit was observed[36].

### Infliximab

Infliximab is a chimeric IgG1 monoclonal antibody against TNF-α (Table 7). Apart from inhibiting TNF-α, recent data suggest that the induction of apoptosis in T cells through infliximab might be an important mechnism of action[37]. Infliximab has shown to be superior over placebo in inducing remission in patients with moderate to severe CD resistance to standard therapy[38]. In this trial, after four weeks 33% of patients went into remission after one single infliximab infusion as compared to 4% of the patients given placebo.

### Summary: Treatment of active CD

In mild and moderately active CD, 5-ASA or budesonide may be used as first line therapy, despite the limited efficacy of 5-ASA shown in randomized, placebo-controlled trials. The presently available budesonide preparations are only efficacious in disease primarily located within the terminal ileum or right colon. In non-responders, systemic corticosteroids should be used. Severe CD should be treated with systemic corticosteroids. If corticosteroids given orally do not lead to improvement, intravenous application should be considered since enteral absorption might be decreased due to severe intestinal inflammation. Enteral nutrition should also be added particularly in malnourished patients (see below chapter on nutrition for details). If an infectious complication is suspected, the additional therapy with antiobiotics (e.g. ciprofloxacin plus metronidazole) might be beneficial. The combination of systemic corticosteroids and azathioprine is superior to prednisolone monotherapy and this combination might be beneficial in severe cases. In patients

refractory to corticosteroids, treatment with infliximab should be considered. Surgery might be neccessary in patients with severe and refractory CD not responding to above mentioned strategies. Intravenous cyclosporin and tacrolimus should only be used in selected severe and refractory cases.

## MAINTENANCE OF REMISSION

Maintaining a medically or surgically induced remission of disease is one of the most important but yet most difficult therapeutic goals in the treatment of CD. Maintenance therapy in CD is characterized as treatment with only a few available drugs, moderately high rates of efficacy and frequent side effects. In total, 40%-70% of CD patients will experience a symptomatic relapse in 1 year after a medically or surgically indused remission[8,9]. Silverstein and colleagues reported that a surgically induced remission lasts a mean of 766 d whereas a non-surgically induced remission lasts only 120 d indicating that a surgically induced remission is more stable[39]. It was frequently recommended that the indication for a relapse-preventing therapy should be based on the prospective risk of an individual patient to relapse. Although the estimation of risk for relapse, based on the phenotype or genotype, is still controversial, single well known risk factors like smoking, frequent relapses in the past, a chronic active disease *etc*. have been described. To stop smoking is a very important therapeutic goal[2,40]. Systemic corticosteroids should not be used for maintaining remission due to lack of efficacy and severe long-term side effects. Since randomized, placebo-controlled trials suggest a different approach in medically or surgically induced remissions, we will handle them separately.

### *Medically induced remission*

**5-ASA:** Numerous randomized, placebo-controlled studies, including four meta-analyses, have attempted to establish a role for 5-ASA in the maintenance of remission. Different study regimens and durations were performed and a substantial number of trials included only small numbers of patients. The two most recent meta-analyses failed to show a benefit for mesalamine over placebo in the maintenance of medically induced remission[41,42]. However, the preferable side effect profile of 5-ASA, especially mesalamine compared to azathioprine/6-mercaptopurine or methotrexate, is probably the reason why mesalamine is still used frequently to maintain a medically-induced remission. Many clinicians therefore try to maintain remission with mesalamine at least one time, especially in young women of childbearing potential. In addition many patients are in favour of trying a rather harmless drug at first for long-term therapy.

**Azathioprine/6-Mercaptopurine:** Azathioprine/6-mercaptopurine is the treatment of choice for patients with high risk of relapse. The effectiveness of azathioprine has been described in a recent meta-analysis including five randomized, placebo-controlled trials. In addition, a steroid-sparing effect was observed[43]. No clear direction has been given as to when to start the treatment with azathioprine/6-mercaptopurine. The following indications are most commonly accepted: frequent flares (more than two per year), chronically active disease, and steroid dependence (e.g. if two attempts of tapering steroids have failed). The thiopurines are slow acting drugs and an effect is usually observed after 2-3 mo with approximately 90% of patients responding within the first 4 mo[17].

An earlier open study suggested that azathioprine is no longer effective after 3.5 years[44]. In contrast, the same group reported, in a very recent placebo-controlled trial, that azathioprine is still effective with prolonged use[45]. However, a small increase in the frequency of malignancy, especially lymphoma, cannot be excluded in the long term treatment with azathioprine/6-mercaptopurine[46,47]. This must be weighed against the improved quality of life due to both drugs for patients with CD.

**Methotrexate:** The potential of methotrexate to induce remission was investigated in a study by Feagan and colleagues. Herein the patients who had achieved remission after weekly 25 mg intramuscularly were randomized to 15 mg methotrexate or placebo. Methotrexate was found to be significantly better than placebo in maintaining remission[26]. However, side effects were more significant than placebo. Methotrexate has not been studied in surgically or medically induced remission by other drugs (e.g. corticosteroids). In summary, methotrexate is suggested to be the alternative to azathioprine/6-mercaptopurine in the maintenance of remission. It has also shown to have steroid-sparing properties with the mean time to respond at about 2 mo.

**Budesonide:** Lower doses of budesonide (3 or 6 mg) have also been studied for their potential to be effective in the maintenance of remission. Although earlier meta-analyses have not shown that budesonide was superior over placebo[16,48], a recent randomized, placebo-controlled trial found a trend towards a longer quiescent disease in budesonide treated patients compared to placebo[49]. In this trial, no significant difference in total adverse events or corticosteroid-associated events was demonstrated between placebo and budesonide. In addition, a very recent paper by Sandborn and colleagues combined the data of four double-blind, placebo-controlled trials with identical protocols analyzing the efficacy of 6 mg budesonide. Budesonide was shown to be effective for prolonging the time to relapse and for significantly reducing the rates of relapse at 3 and 6 mo but not at 12 mo. Herein no difference in the frequency of adverse events and glucocorticosteroid associated side effects between budesonide and placebo was found[50]. Thus, the current data suggest that budesonide at a dosage of 6 mg seems to have the effect of prolonging remission in CD in terminal ileal or right colonic disease. Budesonide might thus offer a potential alternative in the maintenance of a medically-induced remission, especially in steroid-dependent patients.

**Infliximab:** Two studies have shown that infliximab is effective in maintaing remission in CD[51,52]. In the Accent I trial, infliximab was shown to be superior over placebo in the maintenance of remission in CD patients that responded to one single infusion of infliximab. Herein, about 20% of patients in remission after the first infusion of infliximab were maintained in remission for one year with repeated infusions every eight weeks[52]. Infliximab

was also shown to have steroid-sparing properties. Repeated infusions of infliximab should thus be considered for chronically active or steroid-dependent patients where standard immunosuppressants are not effective or where surgical interventions are not considered. However, repeated infusions of infliximab are costly and data on long-term safety, including the occurrence of malignancies, are limited. Infliximab has been shown to lead to mucosal healing, which was associated with reduced surgical interventions and lower hospitalization rates[53]. However, at this time it is debated whether mucosal healing is an important goal in CD therapy. Further studies are warranted regarding this matter. The development of antibodies against infliximab is frequently found and is associated with reduced efficacy and increased numbers of infusion reactions. The concommitant use of immunosuppressants has been shown to reduce the incidence of antibody formation[54].

### *Summary: Maintenance after medically induced remission*

After a medically-induced remission, maintenance therapy should be initiated based on the individual situation. No medical therapy may be considered in patients with low risk of relapse. However, in patients with high risk for relapse (frequent relapses, colonic involvement and severe disease behaviour), therapy with azathioprine or 6-mercaptopurine should be initiated. In patients with terminal ileal or right colonic disease, low-dose budesonide might offer an alternative especially in steroid-dependent patients. In patients who are not responding or are intolerant to azathioprine/6-mercaptopurine, therapy with methotrexate may be used. If not successful, patients should be considered for maintenance treatment with infliximab.

### *Postoperative CD (surgically-induced remission)*

About 75% of CD patients will require surgery within the first 20 years after the onset of symptoms[55,56]. In addition, recurrence rates after surgical resection are high: after the first resection, up to 80% of patients show an endoscopic recurrence within the first year although most patients are not symptomatic[55-57]. Furthermore, up to 20% have clinical symptoms and 5% require another surgical intervention within the first year. After 5 years, about 50% of patients have a clinical relapse. Systemic corticosteroids and budesonide are not effective in preventing postoperative relapse[58-61], whereas methotrexate, ciprofloxacin and infliximab have not been studied for this indication. Various risk factors for postoperative recurrence have been described but most of these risk factors have not been studied in a prospective manner. Currently smoking is the most consistently described risk factor for postoperative relapse[40,62]. In addition, Rutgeerts and colleagues showed that preoperative disease activity and endoscopic lesions at the neoterminal ileum within the first year after surgery are also associated with higher risk for postoperative recurrence[57]. In addition, a recent study suggested that CD patients with CARD15 mutations have a higher risk of postoperative relapse compared to patients without mutated CARD15. Thus genotyping for CARD15 mutations might offer a potential alternative to identify patients with high risk of postoperative relapse[63]. Further studies are warranted to consider this approach.

**5-ASA:** As opposed to the controversial discussion about the efficacy of 5-ASA in the treatment of CD, the results on the prevention of postoperative recurrence are quite solid. Camma and colleagues described in a meta-analysis a risk reduction of 13.1% by mesalamine treatment compared to placebo[41]. A more recent placebo-controlled trial reported that mesalamine did not significantly affect the postoperative course of CD, but some relapse-preventing effect was found in patients with isolated small bowel disease[64]. In summary, 5 ASA is the only treatment with an evidence-based relapse preventing effect after a surgically induced remission and is therefore recommended according to recent guidelines[65].

**Azathioprine/6-Mercaptopurine:** The two largest studies regarding the effect of azathioprine/6-mercaptopurine to prevent postoperative recurrence were recently published. In the first trial, Hanauer and colleagues compared 6-mercaptopurine at the low fixed dose of 50 mg/d to mesalamine 3 g/d and placebo after ileocolic resection[66]. There was a significant benefit of 6-mercaptopurine compared to placebo in preventing clinical and endoscopic recurrence over two years. However, this study has been criticized since it was underpowered and also had a high drop-out rate of patients. Ardizzione and colleagues observed no benefit of azathioprine at standard dosing (2 mg/kg) in preventing clinical relapse after two years in comparison to mesalamine[67]. In summary, although none of these studies offer robust data to support the use of azathioprine/6-mercaptopurine in the prevention of postoperative recurrence, many clinicians use these drugs for this indication.

**Antibiotics:** In a randomized, placebo-controlled trial a significant decrease was observed in the incidence of severe endoscopic recurrence with metronidazole treatment as compared to placebo after ileal resection[68]. In addition, metronidazole therapy statistically reduced the clinical recurrence rates at 1 year. Metronidazole is still only rarely used on this occasion since long term intake is not tolerated by most patients due to side effects such as metallic taste, nausea and peripheral neuropathy. Ciprofloxazin has not been studied in a randomized, placebo-controlled trial regarding the prevention of postoperative recurrence. Rifaximin, which is a non-absorbable drug with good tolerability covering most Gram-positive and Gram-negative bacteria, might offer a very promising alternative since long term application is tolerated much better[69]. At the moment, although frequently used in clinical practice, none of these antibiotics will be considered standard therapy until more controlled trials provide clear results.

Rutgeerts and colleagues investigated the efficacy of ornidazole, a nitroimidazole antibiotic, for the prevention of clinical recurrence after curative ileocolonic resection in a recent placebo-controlled trial. They found that ornidazole significantly reduced the clinical and endoscopic recurrence rate at 1 year compared to placebo. However, significantly more patients in the ornidazole group dropped out of the study because of side effects. In summary, these data indicate that ornidazole might offer a therapeutic alternative in preventing postoperative recurrence[70].

### *Summary: Treatment of postoperative CD*

No standard treatment algorithm prevents postoperative relapse. Despite the controversial discussion on its efficacy, mesalamine over a period of two years is recommended as the treatment of choice in the prevention of postoperative relapse. However, many patients who undergo surgical resection have already been treated with mesalamine so that alternative regimes should be initiated. Although robust data are lacking, most clinicians use azathioprine/6-mercaptopurine at standard dosing in patients with higher risk of postoperative relapse. To estimate the risk of clinical relapse the diagnosis of endosciopic lesions at the anastomosis 6 mo after resection may be used. Azathioprine/6-mercaptopurine may be started if severe or moderate lesions at the anastomosis are found[71]. Although this regime has never been studied in a randomized trial, it seems to be a reasonable approach. Antibiotics, such as metronidazole or ornidazole, might offer a potential alternative although the long term use is limited due to side effects. In addition, prospective studies investigating infliximab in this setting are warranted.

## COMPLICATIONS IN CD

### *Fistulizing disease behaviour*

The treatment of fistulizing CD remains probably the most difficult clinical challenge. Treatment is complicated since very few drugs have proven efficacy whereas most agents used in CD therapy (5-ASA, systemic corticosteroids, budesonide) are ineffective. Fistulae are reported to occur in up to 50% of patients after 20 years of disease[72]. Especially enterocutaneous and enterovaginal fistulae have a severe impact on the quality of live of CD patients. Enterovesical fistulae require surgical intervention due the potential development of an urosepsis. Perianal fistulae are the most common form and are often complicated by an abscess where surgical drainage must be performed. Since complete long term closure of fistulae cannot be achieved in many patients with the available therapies, reduction of fistula drainage and closure of part of the fistulae have been accepted therapeutic goals. Apart from medical treatment approaches as discussed below, surgical interventions such as fistulotomy and insertion of non-cutting setons should be part of the management. A close cooperation between the gastroenterologist and the surgeon is required.

**Azathioprine/6-Mercaptopurine:** Robust data summarized in the meta-anaylsis by Pearson[19] show a positive effect of azathioprine/6-mercaptopurine on fistula closure with an odds ratio of 4.44 (CI 1.50-13.20). Thus azathioprine/6-mercaptopurine is the basis of long-term treatment of fistulae.

**Methotrexate:** No randomized trial has been performed using methotrexate to investigate the healing of fistulae. However, retrospective data showed complete or partial response in 56% (9/16) of patients[73]. Methotrexate might thus be considered as the alternative agent to azathioprine/6-mercaptopurine.

**Antibiotics:** A small uncontrolled study reported a clinical response to metronidazole in 20 out of 21 patients and complete healing after maintenance treatment in 10 out of 18 patients. A follow-up study demonstrated that dosage reduction was associated with exacerbation of fistulae in all patients and healing was again achieved if the drug was reintroduced[74]. Ciprofloxacin alone showed an improvement in 7 out of 10 patients treated with up to 1.5 g over three months[75]. Although controlled clinical trials are lacking, the combination of metronidazole and ciprofloxacin is often initiated.

**Infliximab:** Infliximab offers robust data from randomized, placebo-controlled trials in the treatment of enterocutaneous fistulae. In the first trial by Present and colleagues, three infusions of infliximab at 0, 2, and 6 wk resulted in complete healing of enterocutaneous fistulae in 55% of patients compared to 13% in the placebo group[76]. Data from the ACCENT 2 trial showed that infliximab maintained healing of enterocutaneous fistulae in 36% patients who responded to the initial three infliximab infusions[77]. However, it was shown that healing of fistulae needed repeated infusions, which is similar to the experiences observed in a clinical routine. In summary, data from controlled clinical trials suggest that infliximab might be the most potent drug in the treatment of CD fistulae. Three infusions with a dose of 5 mg/kg at wk 0, 2, and 6 are recommended as standard for the treatment of fistulizing CD.

**Cyclosporin A:** Cyclopsorin A offers an effective alternative treatment for CD fistulae. There are numerous uncontrolled trials that describe a mean initial response in 83% of patients with discontinuation of treatment leading to frequent relapses (reviewed in ref. [78]). However, cyclosporin A toxicity can be dramatic, including renal failure, and thus application should be performed only in centers with expertise. Continuous infusions of 4 mg/kg per day is required and concentrations of 300-400 ng/mL should be maintained[78]. Dosing can be switched to oral if patients respond to intravenous cyclosporin.

**Tacrolimus:** A recent placebo-controlled trial showed that tacrolimus at a dose of 0.2 mg/kg was more effective than placebo in improvement of fistulae (defined as closure of >/= 50% of draining fistulas). However, no difference was observed with respect to fistula remission as defined by closure of all fistulas and maintenance of that closure for at least 4 wk. In addition, adverse events such as headache, increased serum creatinine levels, and insomnia were found significantly more often in the tacrolimus group[79].

### *Summary: Treatment of fistulizing CD*

No standardized treatment algorithm exists in the medical treatment of fistulizing CD. Importantly, effective management requires good collaboration between the gastroenterologist and the surgeon in both simple and complex fistulae. Azathioprine/6-mercaptopurine are the basis of fistulae treatment. Antibiotic combination therapy, preferable with metronidazole and ciprofloxacin, can be considered over a period of 2-3 mo especially if an abscess might be suspected to occur. In patients with complex fistulae including underlying rectal inflammation not improving from above mentioned strategies, a three dose therapy regimen with infliximab should be applied. If patients respond, therapy with azathioprine and infliximab might be necessary to maintain fistula healing. In refractory cases, therapy

with cyclosporine and tacrolimus should be considered.

### *Chronic active disease*

Various definitions of chronic active disease exist and thus results from clinical trials in this complicated group of patients are rather difficult to interpret. The German consensus conference on the treatment of CD describes chronic active disease as the persisting or recurrent occurrence of symptoms over more than 6 mo despite standardized therapy[65]. Patients with chronic active disease should thus be treated first with azathioprine/6-mercaptopurine or alternatively with methotrexate. If patients do not respond or are intolerant to these approaches, infliximab should be given. Due to severe long-term side effects, systemic corticosteroids should be avoided. 5-ASA is not effective in chronically active CD.

### *Steroid-dependent disease*

Steroid-dependency is a frequently observed phenomenon in CD and it is defined as the need for corticosteroids to maintain a patient in stable remission after two unsuccessful attempts to withdraw steroids within the last six months. About 28%-44% of patients will become steroid-dependent after an initial course of corticosteroids[80,81]. Long term use of corticosteroids should be avoided due to severe side effects such as osteoporosis, diabetes and hypertension. Prophylaxis of osteoporosis with calcium and vitamin D should be applied. Similar to patients with chronic active disease, azathioprine/6-mercaptopurine is the treatment of choice and methotrexate is the alternative agent to avoid long term steroid therapy. Two meta-analyses reported a steroid-sparing effect for azathioprine[20,43] and the same properties were observed for methotrexate[26]. In addition, infliximab has also been shown to have steroid-sparing properties and thus should also be considered as an alternative[52].

### *Steroid-refractory disease*

Patients with persisting clinical activity under continuing therapy with corticosteroids at a dose greater than 1 mg/kg per day are described as steroid-refractory. This clinical situation occurs in about 20%-30% of patients treated with corticosteroids[8,9,80]. Only a few drugs have been tested in this situation: azathioprine/6-mercaptopurine and methotrexate have shown to be effective in steroid-refractory patients[20,26,43]. In addition, infliximab offers a therapeutic alternative[52]. However, if medical therapy fails in severe cases, surgical interventions such as colectomy might be necessary.

### *Gastroduodenal CD*

Sympotmatic involvement of stomach and duodenum is a rare phenomenon observed in about 4%-5.5% of patients[82,83]. Endoscopic and histologic involvement might be found in up to 40% of patients[84-86]. Due to the low frequency of patients with symptomatic gastroduodenal involvement, however, randomized, placebo-controlled trials are not available. Combination therapy with high dose acid suppression (proton pump inhibitors) and standard therapy of CD are usually used. Corticosteroids[87], azathioprine[88,89], and infliximab[90] have been reported to be effective in selected patients. However, many patients with obstructive symptoms caused by strictures will have to undergo surgical interventions such as gastroduodenal or gastrojejunal bypass, even performed laparoscopically. Gastroduodenal bypass has been reported to result in a good outcome in up to 87% of patients[91].

### *Fibrostenotic disease behaviour*

CD is often complicated by fibrostenotic strictures that can be located within the whole gastrointestinal tract. Strictures can remain clinically asymptomatic over years until the intraluminal caliber causes obstruction. However, it is often difficult to differentiate between an inflammatory or fibrostenotic stricture. Ultrasound and MRI with the possibility to visualize mucosal blood flow are helpful in differential diagnosis. Before initiating surgical interventions, many clinicians try at least one attempt of medical treatment for strictures suggested to have an inflammatory component. Corticosteroids are most commonly used in this clinical situation. Fibrostenotic strictures will not respond to medical therapy. Endoscopic ballon dilatations, stricturoplasty or resections are required in most cases.

## ROLE OF NUTRITION IN CD

### *Prevention and treatment of malnutrition*

During an acute flare of CD, undernutrition with weight loss, protein deficiency and specific deficiencies in vitamins, minerals and trace elements are commonly found. Malnutrition is mainly caused by anorexia, increased intestinal losses and systemic inflammation. In children and adolescents a decrease in growth velocity may occur, secondary to inadequate nutrition and steroid therapy. The relevance and extent of these deficiencies vary according to the site and extent of the diseased intestine as well as disease activity. In active CD, an improvement in nutritional status cannot be achieved by nutritional counselling alone but oral nutritional supplements or tube feeding leads to improvement of the nutritional status[92,93]. Both malnutrition and growth retardation require enteral nutrition (EN).

The use of oral nutritional supplements or tube feeding should also be taken into account in the perioperative setting. An increased frequency of postoperative complications has been shown in undernourished patients with CD[94], with undernutrition being defined as weight loss and/or plasma albumin levels below 35 g/L. Although specific data concerning the effect of perioperative nutrition in CD are lacking, there is a considerable body of evidence on the effect of perioperative nutrition in general gastrointestinal surgery and preoperative nutritional support is therefore recommended in malnourished patients[95]. A prospective study showed that a preoperative oral supplementation with a formula enriched with arginine, omega-3 fatty acids, and RNA was associated with reduced postoperative infections and shorter lengths of hospital stay[96]. Supplementation of specific deficiencies may be crucial. Iron deficiency is most common and should be treated with oral or i.v. iron supplements. Vitamin D and calcium should be supplemented in patients

on steroid therapy and patients treated with sulfasalazine are at risk to develop Vitamin B12 deficits.

### *Treatment of active disease*

EN is also effective in the treatment of an acute flare in CD with approximately 60% of all patients reaching remission. In children, active disease frequently leads to growth retardation and enteral nutrition is therefore the treatment of choice. In adults, however, treatment with corticosteroids is more effective as shown by a recent meta-analysis[97]. Enteral nutrition as sole therapy for acute CD is indicated mainly when treatment with corticosteroids is not feasible; e.g. due to intolerance or refusal. Combined therapy (enteral nutrition and drugs) is indicated in undernourished patients as well as in those with inflammatory stenosis of the intestine. If active CD is treated with systemic corticosteroids in combination with EN and supplementary EN is continued after the active phase, it prolongs the relapse free interval[98].

Total parenteral nutrition is no better than enteral nutrition in the therapy of active CD and should therefore be restricted to patients with a contraindication to or intolerance of enteral nutrition[99]. EN in subileus and high grade stenosis does require special caution. A documented stenosis however is not a contraindication to EN *per se*[100].

## EXTRAINTESTINAL MANIFESTATIONS

CD is much more than a bowel disease since it can affect almost every other organ of the body. We will describe only briefly the most common extraintestinal manifestations (EIMs) and the recommended therapeutic approaches. The treatment of most extraintestinal manifestations has not arisen from randomized clinical trials but more from experiences and case reports and thus remains often nonempirical. With respect to all EIMs, a collaboration with rheumatologists, dermatologists and especially ophthalmologists should be part of the therapeutic regimen. The basis of treatment of EIMs is to obtain remission since it will positively affect the course of the particular extraintestinal manifestation, especially if symptoms occur parallel to exacerbation of the disease.

### *Arthritis*

Joint involvement is the most frequently found extraintestinal manifestation in CD, which can be separated into axial and peripheral involvement. Peripheral involvement can be subdivided into a pauciarticular, large joint arthropathy, and a bilateral symmetrical polyarthropathy[101]. Axial involvement can result in sacroiliitis or ankylosing spondylitis. Placebo-controlled trials have shown that sulfasalazine is effective in the treatment of ankylosing spondylitis[102,103]. Furthermore physiotherapy is important. A low dose of corticosteroids (usually no more than 10 mg/d) can be a therapeutic option. Nonsteroidal anti-inflammatory drugs (NSAIDs) and COX-II-inhibitors might lead to pain relief but should be avoided since they might exacerbate CD. Many patients need analgetics to control symptoms. The use of tramadol or metamizol is preferable. Due to the experiences with rheumatoid arhritis, methotrexate might be offered as an alternative. In the same respect, infliximab has shown to be very effective[104,105].

### *Erythema nodosum, pyoderma gangrenosum, ocular involvement, PSC*

Erythema nodosum is the most common skin manifestation in conjunction with active CD and usually responds to therapy with corticosteroids. Severe or refractory cases have been shown to respond to infliximab[106]. In pyoderma gangrenosum, corticosteroids are the treatment of choice, even applied by the intravenous route in refractory cases. Topical therapy should be considered as an adjuvant to systemic therapy. However, a recent study reported healing of pyoderma gangrenosum after infliximab treatment in all 13 patients[107]. These results suggest that infliximab might be considered as the treatment of choice for pyoderma gangrenosum, especially in refractory cases. An ocular manifestation such as iridocyclitis or anterior uveitis should be treated with topical steroids and cycloplegics. A case with improvement of uveitis after infliximab treatment was recently reported[108]. Considering primary sclerosing cholangitis (PSC), although more frequently seen in ulcerative colitis, an earlier study showed that ursodeoxycholic acid (UDCA) at a dose of 10-15 mg/d can result in significant liver enzyme improvement[109]. However, a recent 5-year, placebo-controlled trial of high-dose UDCA (17-23 mg/d) failed to show benefit for UDCA on survival or the prevention of cholangiocarcinoma in PSC[110]. Taking all published studies into consideration, Olsson and colleagues conclude that there is, if at all, only a very limited effect of UDCA in PSC. PSC is associated with the occurrence of cholangiocarcinoma where liver transplantation seems to be the only curative approach.

## CONCLUSION

Based on the currently available data from randomized, placebo-controlled trials, including meta-analyses, we describe the conventional treatment of Crohn's disease. This coventional approach suggests a step-up approach usually in the order of 5-ASA, corticosteroids, immunosuppressants and usually infliximab in refractory or severe cases including fistulizing disease behaviour. In contrast, a more aggressive form of treatment (bottom-down) has been recently proposed. This regimen starts out early at diagnosis of CD with the combination of biologicals (infliximab) in combination with immunosuppressants (azathioprine). Studies are warranted to elucidate the role of this new therapeutic approach in comparison to the standard therapy algorithms. Furthermore the value of mucosal healing and its effect on the course of CD, including its potential to reduce complications, surgical interventions and hospitalisation rates, should be evaluated in upcoming studies.

The past years have resulted in enormous new insights into the pathophysiology of CD with respect to molecular genetics, mucosal bacteria and immunology. Now it is time to translate these findings into newer therapeutic concepts. Numerous agents, especially biologicals, have been tested but most of them have not been introduced

into the market due to low efficacy or severe side effects. Apart from infliximab, other TNFα-antagonists, such as adalimumab or CDP870, might offer a potent alternative in the future. However, apart from evidence-based medicine, CD therapy will always be an individualized therapy. In addition, many patients construct their own therapeutic regimen, especially after long term disease. Such approaches might be effective in individual situations, although they do often not stand the criteria of evidence-based medicine. Moreover, many clinical situations are complex and might never have been studied in randomized, placebo-controlled trials. Therefore, the treatment of CD frequently requires individual decisions and creativity despite a very good basis of evidence-based therapies.

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**S- Editor** Pan BR **L- Editor** Lutze M **E- Editor** Liu WF

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*World J Gastroenterol* 2006 August 14; 12(30): 4807-4812
***World Journal of Gastroenterology*** ISSN 1007-9327


*TOPIC HIGHLIGHT*

**Paolo Gionchetti, MD,** *Series Editor*

# Etiopathogenesis of inflammatory bowel diseases

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Received: 2006-02-17 Accepted: 2006-03-10

## Abstract

Theories explaining the etiopathogenesis of inflammatory bowel disease (IBD) have been proposed ever since Crohn's disease (CD) and ulcerative colitis (UC) were recognized as the two major forms of the disease. Although the exact cause(s) and mechanisms of tissue damage in CD and UC have yet to be completely understood, enough progress has occurred to accept the following hypothesis as valid: IBD is an inappropriate immune response that occurs in genetically susceptible individuals as the result of a complex interaction among environmental factors, microbial factors, and the intestinal immune system. Among an almost endless list of environmental factors, smoking has been identified as a risk factor for CD and a protective factor for UC. Among microbial factors, no convincing evidence indicates that classical infectious agents cause IBD, while mounting evidence points to an abnormal immune response against the normal enteric flora as being of central importance. Gut inflammation is mediated by cells of the innate as well as adaptive immune systems, with the additional contribution of non-immune cells, such as epithelial, mesenchymal and endothelial cells, and platelets.



**Key words:** Inflammatory bowel disease; Chronic inflammation; Mucosal immunity; Innate immunity; Adaptive immunity; Environment; Commensal flora

Danese S, Fiocchi C. Etiopathogenesis of inflammatory bowel diseases. *World J Gastroenterol* 2006; 12(30): 4807-4812

http://www.wjgnet.com/1007-9327/12/4807.asp

## INTRODUCTION

It is fair to state most disease entities that still pose major clinical and therapeutic challenges are ones where the exact etiology remains obscure and the mechanisms of tissue injury appear to be exceedingly complex. This certainly seems to be the case for the two main forms of inflammatory bowel disease (IBD); i.e., Crohn's disease (CD) and ulcerative colitis (UC). It is now clear that CD and UC represent two distinct forms of chronic inflammation of the gastrointestinal tract and, as such, have different causes and different pathogenic mechanisms. Still, the factors underlying the appearance of both CD and UC are roughly the same, and include a temporal association with progressive changes in the environment, an intrinsic genetic predisposition, the existence of a rich enteric flora, and an abnormal immune reactivity which is ultimately responsible for damaging the gut and causing clinical manifestations. Even though the categories of underlying factors are roughly the same, there are variations in each category as well as differences in how the underlying factors interact. The end result is two related but distinct disorders named CD and UC. In this review, differences and similarities of the etiopathogenic factors in each form of IBD will be briefly illustrated and discussed.

## ENVIRONMENTAL AND GENETIC FACTORS

A remarkable change in the types of diseases affecting humans has occurred during the last century, most remarkably so in the Western world. The most common illnesses responsible for morbidity and mortality have shifted from infectious to chronic inflammatory and neoplastic diseases. This shift has been best documented in Western countries[1], but the same phenomenon is now occurring in other parts of the world. The emergence of chronic autoimmune and inflammatory diseases, including IBD, throughout the world is closely linked to social and economical progress. This was initially noted in Northern Europe and North America but, after the Second World War, the same phenomenon occurred in the rest of Europe, Japan and South America. Most recently, the emergence of IBD is also being observed in the Asian Pacific Region[2].

The "hygiene hypothesis" has been proposed as the probable underlying reason for the switch from infectious to chronic inflammatory diseases, and it postulates that there has been a fundamental lifestyle change from one with high microbial exposure to one with low microbial

exposure[3]. A relative lack of microbial antigens early in life would lead to a less educated and weaker immune system, not equipped to properly handle new challenges later on in life and generating an ineffective immune response that is prolonged because it is powerless to eliminate the offending agent.

There are innumerable environmental modifications that can be ascribed to the hygiene hypothesis, including better housing, safer food and water, improved hygiene and sanitation, vaccines, the widespread use of antibiotics, lack of parasites, fewer infections, and better but selective nutrition. While contributing to the progressive decline of infectious diseases, at the same time these changes may have contributed to create a surge in allergic and autoimmune diseases[4]. A variety of environmental factors are considered risk factors for IBD, including smoking, diet, drugs, geography and social status, stress, the enteric flora, altered intestinal permeability and appendectomy[5]. Among them, cigarette smoking is the strongest example of the influence of the environment on IBD. Remarkably, smoking has a completely opposite effect on CD compared to UC, indicating that distinct pathogenic mechanisms underlie each form of IBD[6]. Smoking is a recognized risk factor for CD, increasing the frequency of disease relapse and need for surgery, and its discontinuation improves the disease course[7]. Cessation of smoking, however, increases the risk of UC, suggesting a protective role in this form of IBD[8]. Other environmental agents associated with IBD are oral contraceptives and nonsteroidal anti-inflammatory drugs (NSAIDs). These agents have also been investigated as having a cause-and-effect relationship with CD or UC. A direct causal relationship has not been found, but women taking oral contraceptives have twice the risk of developing CD than those not taking contraceptives[9]. In the case of NSAIDs there is a clear association with IBD, and patients in clinical remission have a higher risk of relapse if they use NSAIDs[10].

Although the epidemiological evidence linking environmental factors to IBD is fairly solid, it is widely believed that no environmental factor alone can directly cause CD or UC, and an intrinsic disease predisposition must also be present. Such predisposition depends on genetic susceptibility, and a number of established or potential susceptibility genetic loci have been identified in IBD. This topic will be discussed in greater depth in another chapter of this issue of World Journal of Gastroenterology.

## MICROBIAL FACTORS

### *Pathogens*

It is possible that classical infectious agents are the cause of IBD, but current evidence supporting this hypothesis is rather weak. Over the years, several microorganisms, such as *Listeria monocytogenes*, *Chlamydia tracomatis*, *Escherichia coli*, *Cytomegalovirus*, *Saccharomyces cerevisiae*, as well as others, have been proposed as having an etiologic role. In particular, Mycobacterium paratuberculosis as the agent of CD has received and continues to receive considerable attention. This bacterium is the cause of Johne's disease, a chronic granulomatous ileitis in ruminants that closely resembles CD. M. paratuberculosis was initially isolated from a few CD tissues[11], but follow up studies trying to confirm its presence by histological examination, attempts to culture it from tissue homogenates, search for its genome in intestinal tissues with highly specific probes, and assessment of serum antibodies have all yielded conflicting or inconclusive results. Moreover, controlled trials have failed to show a beneficial effect of antituberculous therapy in CD patients[12]. One of the last bacteria to be linked to CD is an adherent-invasive strain of *E coli* which is specifically associated with ileal CD[13], but its potential etiological role, if any, remains unclear.

The finding of paramixovirus-like particles in CD endothelial granulomas led to the suggestion that CD could be a form of chronic vasculitis caused by the persistence of the measles virus in the mucosa[14]. Based on epidemiological and serologic data, an association between perinatal measles and an increased probability to develop CD was hypothesized[15], but subsequent studies failed to confirm this association. Importantly, the overall decline of measles infection accompanied by the concomitant rise of CD during the last few decades speaks against an etiologic role of measles in CD.

### *Commensal bacteria*

In contrast to the dwindling evidence that CD or UC are infectious diseases, evidence continues to mount that the indigenous commensal flora of the gut is the target of the immune response in IBD[16]. A large body of data from animal models of IBD indicates that the normal enteric flora is needed to develop experimental colitis. In fact, gut inflammation only arises in animals kept in a conventional but not a germ-free environment[17], supposedly because an immune response directed against enteric bacteria is essential to disease pathogenesis[18]. Thus, the paradigm "no bacteria, no colitis" was created to underscore the central role of the intestinal microbiota in IBD pathogenesis. This paradigm is supported by a variety of clinical observations in IBD patients. There is an increased number of bacteria in close contact with the mucosa in IBD patients[19]; IBD lesions occur preferentially in segments with the highest concentrations of bacteria (the ileo-cecal valve and the colon); surgical diversion of the fecal stream prevents reappearance of CD whereas restoration of the fecal flow induces disease recurrence[20]; modulation of the enteric flora with antibiotics and probiotics attenuates inflammation. In addition, pouchitis develops in a considerable proportion of UC patients, and is associated with a dysbiosis caused by the contact of the once near sterile small bowel mucosa with a rich colon-like flora repopulating the pouch soon after proctocolectomy[21].

Finally, most IBD patients show an enhanced systemic and mucosal immunological reactivity against gut bacterial antigens. Among these, based on serum antibody titers, bacterial flagellin has been recently reported as a dominant antigen in CD[22], apparently defining a population of patients with complicated CD[23]. It has been proposed that this immune reactivity is the consequence of a 'loss of tolerance' towards the autologous enteric flora, resulting in an inappropriate immune response in the mucosa which is manifested by the chronic inflammatory process typical

of CD and UC[24]. Under normal circumstances there is an intimate interaction between commensal intestinal bacteria and the immune system[25], and this complex crosstalk is under the control of immune tolerance[26]. Why tolerance is lost and an abnormal response to otherwise normal gut bacteria develops in IBD is still not entirely clear. However, the recent discovery that CD is genetically associated with mutations of the NOD2/CARD15 gene, whose product is a bacteria-recognizing cytoplasmic protein, points to defective mechanisms of bacterial sensing as the link between the gut flora and the altered immune response found in IBD[27].

## CELLULAR FACTORS

The most common type of reaction that the body mounts against external or internal offending agents is inflammation. The gut is particularly susceptible to inflammation as indicated by the fact that, and even under normal circumstances, there is a baseline degree of "physiological inflammation" in the mucosa. This is caused by a tightly controlled immune response directed at an enormous array of dietary and microbial antigens, and it is translated by the presence of an abundant number of leukocytes in the lamina propria[28]. The ultimate goal of an effective inflammatory response is to eliminate the offending agent(s) and then disappear once the cause of inflammation has been eradicated. If inflammation persists and becomes chronic, it represents an inappropriate response that almost invariably leads to lingering injurious effects resulting in anatomical and functional abnormalities. Both CD and UC are typical chronic inflammatory processes of the gut which, by definition, are due to abnormalities of the intestinal immune system. Fortunately, major advances have occurred during the past three decades in our understanding of the cellular and molecular mechanisms mediating mucosal immunity and the alterations that lead to chronic gut inflammation[29].

### *Adaptive immunity*

Abnormalities of intestinal immunity in IBD began to be described several decades ago in regard to the main effector cells of adaptive immunity; e.g., T-and B-cells. Initially, it was discovered that the production of antibodies, particularly IgG antibodies, in the systemic as well as mucosal compartments was drastically increased and that the relative proportions of immunoglobulin classes and subclasses were altered as a consequence of chronic gut inflammation[30-32]. In parallel with these studies, the possibility that some of these antibodies were true autoantibodies directed at self-components of the gut began to be explored. A series of studies suggested that IgG1 antibodies against a structural protein of colonocytes were selectively produced in UC, but not in CD, and could underlie the pathogenesis of this condition[33]. Until now, however, definitive proof for the existence of classical, tissue injury-inducing autoantibodies in UC is still missing. With the recognition of T-cells as central effector cells and their soluble mediators as key modulators of immunity, the focus of immune investigation in IBD shifted to T helper (Th) cell subsets and the soluble mediators they produce. A large number of cytokine abnormalities have been described, including pro-inflammatory and immunoregulatory molecules[34]. In CD, intestinal CD4+ T cells produce large amounts of INF-γ and display marked overexpression of the Th1-cell-specific transcription factor, T-bet[35], while mucosal macrophages produce large amounts of IL-12 and IL-18[36,37]. Additionally, CD mucosal T-cells are resistant to apoptosis and cycle faster than control cells[38,39]. In contrast, in UC nonclassical CD1d-restricted NK T-cells produce increased amounts of IL-13, and mucosal T-cells produce more IL-5, cycle slower and die more than control cells[39-41]. Based on these observations, it is now generally accepted that the two main forms of IBD are associated with distinct immune profiles which are classified as a fairly typical Th1 response in CD and an atypical Th2 response in UC.

More recently, the study of adaptive immune abnormalities in IBD has been focusing on possible defects of immunoregulation. Different types of immunoregulatory cells exist, the best defined being CD4 + CD25 high T-cells, which are critically important in preventing autoimmunity and suppressing excessive immune reactivity[42]. In IBD there is a contraction of this regulatory cell pool in the blood and only a moderate expansion in the inflamed intestine, suggesting the presence of insufficient regulation during active disease[43].

### *Innate immunity*

With the discovery of an association of a group of CD patients (those with small bowel and stricturing disease) with mutations of the NOD2/CARD15 gene, whose product is found in cells mediating innate immunity (primarily macrophages and dendritic cells) and recognizes the bacteria-derived component muramyl dipetide (MDP)[44,45], a surge of interest in the role of innate immunity in IBD has occurred. Dendritic cells are scarce in the gut mucosa, but form a heterogeneous population of potent antigen-presenting cells pivotal to the balance between tolerance and active immunity and controlling the type of response - inflammatory or not - that follows detection of commensal bacteria[46]. In IBD, mucosal dendritic cells are activated, express increased levels of the toll-like receptors (TLR) 2 and TLR4- which mediate recognition of bacterial products - and CD40, and produce more IL-12 and IL-6[47]. All of these phenotypic and functional features indicate a prominent role of dendritic cells in IBD pathogenesis. Epithelial cells are also involved in innate immunity. Interestingly, ileal Paneth cells also express the NOD2 protein, and their production of mucosal α-defensins is decreased in CD patients with NOD2 mutations, perhaps leading to an impaired resistance against enteric microorganisms and eventually contributing to bacteria-induced inflammation[48].

Another crucial component of innate immunity is the TLRs, cell surface molecules that detect microbial infection and trigger antimicrobial host defense responses[49]. TLRs are abundantly expressed on the surface of monocytes, macrophages, and dendritic and epithelial cells and, in addition to recognizing pathogenic microorganisms, are essential to identify the commensal microflora and maintain intestinal homeostasis[50]. Alterations of TL3 and

TLR4 expression by intestinal epithelial cells have been described in IBD, suggesting the possibility that abnormal bacterial sensing contributes to disease pathogenesis[51]. Because both NOD2 and TLRs are involved in innate immunity and recognition of and response to bacteria, much attention has been recently devoted to their biological interrelationship and the possibility of functional abnormalities in IBD, and CD in particular. Monocyte-derived macrophages of CD patients carrying homozygous mutations of NOD2 show clear-cut defects of IL-1β and IL-8 production upon activation by MDP or TNF-α[52]. Moreover, the synergism between MDP and TLR ligands that causes a substantial upregulation of TNF-α and IL-1β production in normal peripheral blood mononuclear cells is lost using cells from CD patients with double mutant genotypes[53]. Thus, these preliminary reports point to the existence of generalized major defects of innate immune responses mediated *via* pattern recognition receptors in CD.

### *Nonimmune cells*

Other cell types participate in the chronic inflammatory response of IBD, including epithelial, mesenchymal and endothelial cells, and platelets, which actually exert many of the functions traditionally attributed to classical immune cells, such as cytokine production or expression of MHC class II antigens.

Initial evidence that intestinal epithelial cell (IEC) function may be altered in IBD was acquired when immunohistochemical studies showed that IEC inappropriately expressed the class Ⅱ antigens HLA-DR in actively inflamed mucosa of UC and CD patients[54]. Later on, after the demonstration that normal IECs have antigen-presenting capacity and preferentially stimulate CD8+ suppressor T-cells, a report showed that IEC from IBD mucosa fail to induce such cells and instead activate CD4+ T-cells, and thus potentially amplify intestinal inflammation[55]. More recently, IBD IECs were reported to inappropriately express members of the B7 family of co-stimulatory molecules[56], a finding suggesting possible alterations in B7-ICOS costimulatory pathways in IBD. These reports, together with the above-mentioned altered expression of TLRs in IBD[51], provide support for the notion that IECs have a role in IBD pathogenesis, but to fully understand their functional relevance will require additional investigation.

The involvement of fibroblasts in IBD has been traditionally viewed as one restricted to production in the extracellular matrix and the pathogenesis of a common and serious complication; e.g., intestinal fibrosis[57,58]. However, fibroblasts are also involved in gut injury because they represent a major source of matrix metalloproteinases (MMPs), a family of proteolytic enzymes directly responsible for tissue destruction during inflammation[59,60]. Of special importance is the observation that interaction with activated T-cells is a major pathway of fibroblast activation and MMP production, a phenomenon that links together fibroblast function, adaptive immunity, and gut tissue injury[61]. In reality, the functional interaction of mucosal fibroblasts with the surrounding microenvironment is physically more complex and functionally more important than previously recognized. A recent report showed that activation of fibroblasts through the CD40 pathway induces the upregulation of cell adhesion molecules and production of chemokines which, in turn, induce the migration of T-cells through local microvascular cells[62]. Therefore, mucosal fibroblasts must also be considered as active rather than passive participants in IBD pathogenesis.

Endothelial cells play an essential role in inflammation due to their central "gatekeeper" function, which controls the quality and quantity of leukocytes that transmigrate from the vascular into the interstitial space. This process is complex and is mediated by a number of molecules, including cytokines, chemokines and adhesion molecules. A key observation that opens the whole field of functional vascular biology in IBD is that human intestinal microvascular endothelial cells (HIMEC) isolated from CD and UC mucosa exhibit a significantly higher cytokine-mediated leukocyte binding capacity compared to HIMEC from normal mucosa[63], a phenomenon secondary to their chronic exposure to the inflammatory milieu of the IBD mucosa[64]. Increased leukocyte adhesion by IBD HIMEC is apparently due to their deficient production of inducible nitric oxide (NO) synthase[65]. This also causes a microvascular endothelial dysfunction in IBD due to a loss of NO-dependent dilation that may lead to reduced perfusion, poor wound healing, and maintenance of inflammation[66].

The role of platelets in IBD has been known for quite some time, but primarily because of their involvement in thrombotic events which are relatively common in CD and UC patients[67]. However, platelets have increasingly acquired a strong immunological connotation through the demonstration of their initiator or amplificatory role in immunity and inflammation, which is mostly mediated through the CD40/CD40 ligand pathway[68]. Platelets exist in an activated state in the peripheral circulation of IBD patients, and the elevated levels of soluble CD40 ligand present in their systemic circulation are mostly of platelet origin, apparently due to platelet activation in the inflamed intestinal microvascular bed[69]. More importantly, recent studies have shown that platelets trigger a CD40-dependent inflammatory response in the microvasculature of IBD patients[70], thus closely linking this unique cell type to the process of IBD pathogenesis.

## CONCLUSIONS

Since the recognition of IBD as a perplexing and challenging clinical entity, the investigation of its pathogenic mechanisms has gone through repeated cycles of new hopes, new knowledge, and new realities. Infectious, allergic, dietary, psychosocial, environmental, microbial, vascular, metabolic, immune and other based theories have been put forward, most of them to be rebuked, if not ridiculed[71]. At the moment, we appear to have settled down on a unifying but still wide-ranging hypothesis that IBD results from complex interactions between evolving environmental changes induced by society's progress, a still undefined number of predisposing genetic mutations, an incredibly complex gut microbiota that may be constantly varying, and the intricacies of

individual immune systems[72]. The ability to integrate all these various components into a single cohesive and logical pathway of disease that explains all aspects of IBD appears still a bit distant at the moment. On the other hand, if we look back at where we stood only two or three decades ago, the progress achieved in our understanding of IBD pathogenesis and the way it has changed our approach to therapy is just short of spectacular.

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**S- Editor** Pan BR **L- Editor** Zhu LH **E- Editor** Ma N

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*World J Gastroenterol* 2006 August 14; 12(30): 4813-4818
***World Journal of Gastroenterology*** ISSN 1007-9327


*TOPIC HIGHLIGHT*

**Paolo Gionchetti, MD,** *Series Editor*

# Cytomegalovirus and inflammatory bowel disease: Is there a link?

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Received: 2006-02-17 Accepted: 2006-03-10

## Abstract

The objective of this report is to give an overall view of the epidemiological, clinical, diagnostic and therapeutic features of Cytomegalovirus (CMV) infection in inflammatory bowel disease (IBD). A review of published reports on this topic was carried out, with particular attention paid to the selection of patients included in studies and the diagnostic methods employed. CMV is frequently associated with IBD. In some cases, CMV infection is associated with a poor outcome but it is not clear which patients are more likely to be affected and in which stage of the disease. The use of anti-viral therapy in IBD is controversial and an empirical study with controls is needed. The natural history of CMV infection related to the development and treatment of IBD has not been clarified but it is important to take it in consideration because of the possibility of viral persistence in the immunocompromised host and viral interaction with the immune system.



**Key words:** Cytomegalovirus; Inflammatory bowel disease; Outcome

Criscuoli V, Rizzuto MR, Cottone M. Cytomegalovirus and inflammatory bowel disease: Is there a link? *World J Gastroenterol* 2006; 12(30): 4813-4818

http://www.wjgnet.com/1007-9327/12/4813.asp

## INTRODUCTION

Cytomegalovirus (CMV) is a member of the Herpesviridae family, which includes Epstein -Barr virus (EBV), Herpes Simplex virus tipes1 and 2 (HSV-1,2), Varicella- Zoster virus, and Human Herpes virus types 6 and 7 (HHV-6,7). Similar to infection with other viruses in the family, primary infection with CMV results in the establishment of a persistent or latent infection, due to the ability of the virus to remain integrated in the DNA of host cells. The sign of a viral infection is a cytopathic effect shown by the presence of large nuclear and cytoplasmic inclusions, represented by aggregates of replicating CMV nucleoprotein cores. To avoid recognition and destruction by CD8+T lymphocytes, the virus develops the ability to evade the immune system by several mechanisms. CMV produces some proteins, such as US2, US3 and US11, that inhibit the presentation of viral antigens to T cells, blocking the class I MHC in the endoplasmic reticulum or in the cytosol. It also produces homologues to class I MHC proteins and may compete for binding and presentation of viral antigens. Once the viral DNA remains undisturbed in the infected cells, subsequent reactivation can occur in response to several stimuli, such as immunosuppressant therapy or chemotherapy[1]. The down-regulation of the cell surface markers acts on interferon-alpha/beta dependent responses by affecting several levels of IFN signal transduction and a transcription activation pathway. CMV infection leads to the activation of Nuclear Factor kappa B (NF-κB) and its translocation to the nucleus, promoting the expression of cytokines, chemokines and cellular adhesion molecules. These mechanisms are also present in inflammatory bowel disease (IBD) where there is activation of several pro-inflammatory cytokines (such as IFN-gamma), the transcription of which is regulated through nuclear transcription factors (such as Nuclear Factor kappa B) and through a signal transducer and activator of transcription (STAT family).

## CMV AND IBD

The role of CMV in IBD is reviewed considering the following issues: diagnostic methods to detect the virus, prevalence of CMV in IBD according to patient selection, clinical characteristics, outcome for patients with IBD and superimposed CMV infection, role of antiviral therapy and natural history of CMV in IBD patients.

### *Diagnostic method*

Results differ based on diagnostic method. Serology is

useful in checking for previous virus exposure and for identifying patients at risk. Since gross appearance is non-specific, a diagnosis is based on the histopathological identification of viral-infected cells in biopsied tissues, using appropriate staining Haematoxylin and Eosin (HE), Immunohistochemistry (ICH), by a dedicated pathologist. Cytomegalic cells have been revealed that are 2-or 4 fold larger (25-35 μm) than surrounding cells, containing a basophilic intranuclear inclusion (8-10 μm) that is eccentrically placed and is sometimes surrounded by a clear halo, giving it an "owl's eye" appearance. These cells also show a thickened nuclear membrane, frequently associated with smaller granular intracytoplasmatic inclusions.

Intranuclear inclusions were observed in epithelial, endothelial, stromal and smooth muscle cells.

In the gastrointestinal tract, "atypical" inclusions were also found[2]. Biopsies taken from the mucosa near or within the ulcer provided greater detection of the virus. IHC is more sensitive than HE.

The CMV pp65-antigenemia assay as Polymerase Chain Reaction (PCR) DNA amplification is a sensitive, specific and rapid method for the early diagnosis of CMV infection based on immunocytochemical detection of a virus protein (pp65) in the nuclei of peripheral blood polymorphonuclear leukocytes. The advantage of this method is an early and rapid response possibility but this technique does not permit the distinction between an asymptomatic infection and an active disease. Viral load quantification may permit the observation of the infection course. PCR is a very sensitive test for the detection of CMV. In solid organ transplant patients, CMV detection can persist for months using qualitative PCR assays, despite effective antiviral therapy. Therefore, the precise amount of CMV DNA may be better determined with virological monitoring. In addition to studies using quantitative PCR in renal transplant recipients, studies on peripheral blood leukocytes suggest a cut-off of > 1000 copies/100 000 leukocytes, indicative of the development of symptomatic CMV infection after transplant[3]. A slow or absent decline in CMV DNA after the beginning of ganciclovir therapy could be an early indicator of drug resistance[4]. A PCR positive assay after ganciclovir therapy, irrespective of resolution of clinical signs and symptoms, might be an indicator that therapy should be continued[5]. Mendez *et al*[6] analyzed the early diagnosis of CMV infection using four sets of primers that were able to amplify different regions of the CMV genome. The authors demonstrated that a specific primer directed to the HIND III-X fragment region is the most sensitive primer for the early detection of CMV DNA in peripheral blood leukocytes (PBLs), providing detection 17 d before the onset of the symptoms. *In situ* hybridization and qualitative PCR in colonic biopsies seem to offer the greatest accuracy[7-9] in detecting the virus.

Quantitative PCR (viral-load) in cells from the colon[10] showed a high positive-predictive value for detecting disease and for monitoring therapeutic response. A qualitative and quantitative PCR assay for CMV DNA has been performed on human faecal specimens from immunocompromised patients[11,12].

### *Patient selection method*

The reports on prevalence of CMV are extremely varied in regard to patient selection methodology. There is no study that gives an overall prevalence of CMV in IBD patients. Most of the studies have been carried out using a selected patient group (severe colitis, steroid-resistant colitis, urgent colectomy for colitis, patients with active disease) and different diagnostic methods were used as well for different patient group (e.g., histology, ICH, antigenemia, electron microscopy, *in situ* hybridization). Prevalence has been reported to range from 0.5 to 100% (Table 1).

**Severe colitis:** A group in Italy[13] studied the prevalence of CMV using rectal biopsies and HE staining, immunoperoxidase staining for CMV antigens and antigenemia pp65 (a buffy-coat preparation) on the peripheral leukocytes. The study was performed prospectively in a consecutive series of hospital admissions without waiting for a possible response to conventional therapy, in order to determine the prevalence of CMV in severe consecutive episodes of colitis. The results showed virus prevalence overall in patients with active IBD (21%). On the basis of these results the authors suggested performing a flexible proctoscopy (without air insufflation) with rectal biopsies in patients hospitalized for severe colitis flare-up, together with antigenemia on peripheral leukocytes, to determine whether the simultaneous detection of virus both in the colon mucosa and in the peripheral blood may be interpreted as the pathogenic cause.

Khishore[14] used serology, HE and PCR for CMV DNA to study a heterogeneous population, including patients with severe colitis. Thirty-six patients had severe colitis, with 8 patients (22%) shown to be CMV positive based on colonic biopsies. Moreover, the author identified clinical variables associated with a higher risk of CMV infection in IBD. These factors included female gender, pancolonic disease with active inflammation at histology, and azathioprine treatment. Using antigenemia, Wada[15] reported a prevalence of 34%, while Vega[16] reported a result of 3% in a retrospective study that used HE and ICH results.

**Severe steroid-resistant colitis:** Cottone *et al*[17] showed that the prevalence of CMV, studied using HE and ICH and antigenemia, was 36%. Kambham[18] obtained a similar result, using HE and ICH on colonoscopic biopsy specimens, detecting CMV in 4 of 15 steroid-resistant patients (26%). Pofelsky[19], using PCR, showed a higher prevalence (60%) compared to previous reports, and an overall prevalence of viral DNA in the colon of 38%. However, there was a poor correlation between colonic and peripheral viral load which suggests a role of local inflammation in the colon. The genotype detected was gB1, which possibly has a particular colonic tropism. On the contrary, Papadakis[20], in a retrospective study on 1895 patients, showed a low rate of 0.5% prevalence. He suggested a pathogen role for the virus based on the prompt response and clinical improvement found with antiviral treatment.

**Urgent colectomy for colitis:** These patients represent a subgroup in which IBD is more severe and, therefore, a higher prevalence of CMV is expected. Six studies

**Table 1 Prevalence data according to population and methodology of the study**

| Author (year of publication) | Population characteristics | Diagnostic assay | Study type | Prevalence |
|---|---|---|---|---|
| Criscuoli (2004) | 42 pts with severe colitis | HE-ICH on rectal biopsy + antigenemia pp65 | prospective | 21% |
| Kishore (2004) | 63 pts with severe colitis (36 steroid resistant) | Serology-HE and qualitative PCR | prospective | 16%<br>22% |
| Wada (2003) | 47 pts with severe colitis | Antigenemia | prospective | 34% |
| de Saussure (2004) | 64 pts with active colitis | Serology-viremia-antigenemia- HE-ICH | prospective | 1.5%-6% |
| Vega (1999) | 267 active colitis | HE-ICH | retrospective | 3% |
| Pofelski (2005) | 48 pts severe colitis | Quantitative PCR on colonic biopsies | retrospective/<br>prospective | 38%<br>60% |
| Kambham (2004) | 40 pts steroid resistant (25 operated on for colectomy) | HE-ICH | retrospective | Overall 25%<br>Operated on pts 24% |
| Papadakis (2001) | 1895 steroid resistant pts | HE | retrospective | 0.5% |
| Cottone (2001) | 19 Steroid resistant pts | HE-ICH on rectal biopsy + antigenemia pp65 | prospective | 36% |
| Maconi (2005) | 77 pts operated on for colectomy | HE-ICH on surgical specimen | prospective | 22% overall<br>27% steroid resistant |
| Takahashi (2004) | 69 surgical specimen of IBD pts | HE-ICH on biopsy and surgical specimen | retrospective | 11.5% |
| Alcalà (2000) | 39 pts operated on for colectomy | HE + ICH | retrospective | 18% |
| Eire-Brook (1986) | 26 pts operated on for colectomy | Light and electron microscopy- ICH | retrospective | 11.5% |
| Cooper (1977) | 46 pts operated on for colectomy | HE | retrospective | 13% |
| Rahbar (2003) | 23 pts with IBD (13 UC-10 CD) | ICH+ *in situ* hybridization | prospective | 92/100% |
| Wakefield (1992) | 50 pts with IBD (29 CD-21 UC) | qualitative PCR | prospective | 66/81% |

(two older studies using HE and four recent studies using ICH) have been reported on surgical specimens from patients with colitis who were not responsive to medical therapy[6,21-25]. These studies showed an overall prevalence (11.5%-27%) that is similar to reports in other studies. In the subgroup of steroid-resistant patients, Kambham and Maconi reported a similar prevalence of 25%-27%.

**Patients with active disease:** Rahbar[7] has estimated virus prevalence in intestinal biopsies from IBD patients both using ICH and *in situ* hybridisation. The latter showed detection in over 90% of ulcerative colitis (UC) patients, and in 100% of patients with Crohn's disease (CD). Since the presence of the virus does not necessarily mean active infection, the authors looked at viral replication by CMVea (early antigen) and CMVla (late antigen) by immunohistochemistry, and they obtained a similar result (85% *vs* 100% for UC *vs* CD, respectively) using CMVea.

Other authors have used an immunoperoxidase technique, using a monoclonal antibody against CMV, to demonstrate early CMV infection in cells of colonic specimens in cases showing few cytopathic cells at histological examination. On the contrary, the DNA *in situ* hybridization technique was less helpful in establishing a diagnosis of early infection[8].

A study that used PCR showed positive detection in about 66% of CD patients, 81% in UC patients, and 29% in controls[9].

### CMV and acquired immunodeficiency syndromes or immunosuppressive therapy

The gastrointestinal system is a common site of CMV infection, especially in AIDS patients. Any tract can be affected, with preference to oesophagus and colonic mucosa (especially right colon) rather than ileum, considering the pouch mucosa are morphologically similar to colon mucosa[26]. The syndrome begins with a watery diarrhoea due to an inflammatory response, that quickly turns into bloody diarrhoea due to ulcerative changes in the colonic mucosa. The endothelial cells are a site of CMV detection both in a latent state and active replication state[27]. This explains why CMV vasculitis is a common manifestation of viral disease localized in different organs (bowel, retina, brain) and that vascular damage is responsible for thrombosis[28] and atherosclerosis. In the bowel, vasculitis may cause ischemia and transmural necrosis with an increased risk of toxic megacolon and perforation.

A recent meta-analysis[29] on the outcome of CMV colitis in an immunocompetent host reviewed 44 cases (of which 16 had coexisting immune-modulating morbidities such as diabetes, malignancy or renal failure). The conclusion of this analysis was that CMV colitis is found more frequently in elderly patients in whom the disease had a severe course and where there was a high mortality rate. On the contrary, younger patients (< 55 years) had a significant rate of spontaneous recovery, with 42% of patients diagnosed with subsequent IBD after resolution of CMV infection. Ng[30] carried out a retrospective analysis on patients without apparent causes for immunodeficiency but who were mainly elderly and were admitted to hospital with bloody diarrhoea. The mortality rate reported was 40% which was thought to be related to co-morbidity

**IBD and superimposed CMV infection:** When the syndrome appears with a pre-existing inflammatory disease, IBD may be more aggressive. The clinical outcome of IBD with superimposed CMV is not well understood. The first report about the possible role of cytomegalovirus in IBD dates to 1961 when Powell[31] described a case of ulcerative colitis and cytomegalic inclusion disease. After many sporadic reports over the last decade, the topic has

regained attention due to more frequent publications of case-reports or small series studies in which the virus provided a worsening prognosis influence, sometimes promoting disease initiation or otherwise acting as a bystander[9].

The coincidental detection of primary CMV infection at the first appearance of IBD is reported in some case-reports[32,33], underlining the ability of CMV proteins to enhance pro-inflammatory cytokines that are able to maintain a local colonic inflammation with an immune response. In other conditions the latent virus could exacerbate[34-36] pre-existing colitis after immunosuppressive situations. Experimental studies have shown that highly proliferating cells, like those in the granulation tissue, around inflammation or in ulcer depth, are easily objects of CMV infection. In this situation the virus could reach the mucosa by monocytes and then colonize the mucosa, acquiring particular affinity for the inflamed mucosa.

This is evidenced by the fact that super-infection with pre-existing colitis causes worsening of symptoms, with a severe course of disease that rarely strikes suddenly, with high prevalence of toxic megacolon and surgical intervention[10,11,33]. In this case, since steroids or immunosuppressive therapy can lead to the flare-up of CMV infection, the outcome for patients with an acute attack is likely to be poor. A case of disseminated (whole gastrointestinal tract, skin and central nervous system) CMV infection has been reported in Crohn's disease after anti-TNF therapy[37]. The mechanism of dissemination is likely to be related to vascular damage that allows a viral circulation within the shed endothelial cells.

The literature contains many case reports of CMV infection in steroid-naïve patients or immunocompetent hosts with IBD. Rachima[38] reported two cases of CMV infection diagnosed by high titres of IgM antibodies to CMV (solid-phase enzyme immunoassay) but without histological detection on colonic biopsies. A high prevalence of CMV IgG antibody in patients with ulcerative colitis, compared to normal controls and to patients with active Crohn's disease, permits the hypothesis of a possible role for the virus to exacerbate the inflammatory disease and therefore its pathogenicity. Although clinically significant CMV infection occurs in individuals with acquired immunodeficiency syndrome or due to immunosuppressive therapy, the same inflammatory disease could be considered a booster of viral infection with local factors, even without previous immunosuppressive therapy.

**Antiviral treatment:** A review of the literature does not affirm that antiviral treatment is mandatory when CMV is detected in biopsy specimens or in peripheral blood, however, some authors are favourable to the use of antiviral treatment[2-9]. Eddleston[39] proposes the consideration of antiviral therapy in immunocompetent patients with multiple organ disease, taking into consideration the poor prognosis with widespread CMV disease. Pfau considers Ganciclovir as a beneficial treatment that significantly decreases the mortality rate and the request for surgery. Eire-Brook considers CMV as a bystander and refuses to allow any antiviral treatment during an acute flare-up of IBD.

It is difficult to draw conclusions about the role of antiviral therapy on the basis of the available evidence. Severe steroid-resistant colitis is the setting in which the role of antiviral therapy should mainly be considered. According to some reports, antiviral therapy treatment was useful, whereas in others it was useless. A controlled study is necessary to answer this question, however a large number of patients would be needed for such a trial and it would therefore be necessary to involve many centres using homogeneous diagnostic methods.

**Natural history of CMV infection:** No studies have reported on the natural history of CMV infection. It is not known whether the virus remains in the colon after an acute attack or spontaneously disappears. It is also not known whether there is a high incidence of relapse when the virus remains in the colon. Furthermore, it is not clear whether the virus plays a role in risk for cancer and lymphoma of IBD. For many years, the involvement of Human Herpes Virus (EBV, HHV-8) has been well known in the pathogenesis of some tumours, while there is little knowledge about the potential oncogenetic role of CMV.

A study performed using ICH, *in situ* hybridisation and PCR on pathological specimens of colorectal polyps, adeno-carcinomas and normal mucosa, reported an immunoreactivity of at least 80% in the colorectal polyps and carcinoma both for an immediate early gene product (IE1-72) and for a delayed early gene product (pp65), and acid nucleics. Moreover, detected cyclo-oxigenase -2 in the same site of virus detection may play an important oncogenetic role in the development of human colorectal cancer. The virus could activate cellular proto-oncogenes, kinases and transcription factors implicated in tumour-cell survival pathways[40].

On the contrary, a more recent study[41] using similar methods did not find nuclear immunoreactivity for CMV proteins and DNA, but found focal cytoplasmic positivity in normal colonic mucosa as well, demonstrating no evidence of association. Adani[42] reported a case of severe ulcerative colitis with superimposed colonic CMV infection without peripheral involvement that was associated with colorectal high-grade B-cell non Hodgkin's lymphoma (MALT type). More frequent association is reported between CMV pneumonitis and non-Hodgkin's lymphoma. Both cases were associated with previous immunosuppressive treatment, which is a well-known promoting factor.

## CONCLUSION

In general the role of CMV in IBD remains unclear. On the basis of the current evidence,in our opinion, in active severe IBD CMV, if is detected in the colonic biopsies together at the presence of antigenemia, should be treated with antiviral drugs. There is certainly a pathogenic role of CMV in immunosuppressed transplant patients and in patients with AIDS where treatment is mandatory.

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**S- Editor** Pan BR **L- Editor** Lutze M **E- Editor** Liu WF

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*World J Gastroenterol* 2006 August 14; 12(30): 4819-4831
***World Journal of Gastroenterology*** ISSN 1007-9327


*TOPIC HIGHLIGHT*

**Paolo Gionchetti, MD,** *Series Editor*

# Extraintestinal manifestations and complications in inflammatory bowel diseases

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Received: 2006-05-18 Accepted: 2006-06-20

## Abstract

Crohn's disease (CD) and ulcerative colitis (UC) are chronic inflammatory bowel diseases (IBD) that often involve organs other than those of the gastrointestinal tract. These nonintestinal affections are termed extraintestinal symptoms. Differentiating the true extraintestinal manifestations of inflammatory bowel diseases from secondary extraintestinal complications, caused by malnutrition, chronic inflammation or side effects of therapy, may be difficult. This review concentrates on frequency, clinical presentation and therapeutic implications of extraintestinal symptoms in inflammatory bowel diseases. If possible, extraintestinal manifestations are differentiated from extraintestinal complications. Special attention is given to the more recently described sites of involvement; i.e. thromboembolic events, osteoporosis, pulmonary involvement and affection of the central nervous system.



**Key words:** Inflammatory bowel diseases; Crohn's disease; Ulcerative colitis; Extraintestinal manifestations; Complications; Therapy

Rothfuss KS, Stange EF, Herrlinger KR. Extraintestinal manifestations and complications in inflammatory bowel diseases. *World J Gastroenterol* 2006; 12(30): 4819-4831

http://www.wjgnet.com/1007-9327/12/4819.asp

## INTRODUCTION

Crohn's disease (CD) and ulcerative colitis (UC) are the main entities of chronic inflammatory bowel diseases (IBD). Although in most cases the gastrointestinal tract is mainly affected, both ulcerative colitis and Crohn's disease are systemic disorders that often involve other organs. These nonintestinal affections are termed extraintestinal *symptoms* and may not always coincide with the underlying bowel disease. Extraintestinal disease can involve almost every organ system. The organs most commonly involved include the skin, eyes, joints, biliary tract and lungs. Some symptoms, such as oral lesions, gallstones, pancreatitis, nephrolithiasis and amyloidosis, are more associated with CD than with UC. Other symptoms, e.g. skin and eye manifestations, are equally seen in both CD and UC.

Several factors may be responsible for extraintestinal organ involvement in IBD and sometimes it can be difficult to differentiate the true extraintestinal *manifestations (EIMs)*; i.e. primary systemic affection by the disease itself, from secondary extraintestinal *complications* of the disease, caused for example by malnutrition, chronic inflammation or side effects of therapy. Some of these EIMs may not correlate with disease activity (primary sclerosing cholangitis and ankylosing spondylitis) but in general EIMs tend to follow the clinical course of IBD and may have a high impact on quality of life, morbidity and even mortality in these patients.

The reported frequency of EIMs in patients with IBD varies from 6%-47%[1-4]. The development of one EIM appears to increase the susceptibility of developing other EIMs. An overlap of EIMs is particularly observed with peripheral arthritis, erythema nodosum, affection of the biliary tract and the eyes, in concordance with the hypothesis of a common pathogenic pathway. Some authors discuss an autoimmune reaction towards an isoform of tropomyosin (Tropomyosin related peptide), which is expressed in eye (non-pigmented ciliary epithelium), skin (keratinocytes), joints (chondrocytes), biliary epithelium and the gut[5,6].

The high concordance in EIMs in siblings and first degree relatives with IBD[7] suggests a common genetic background. Crohn's disease and ulcerative colitis are polygenic disorders and certain susceptibility genes in the major histocompatibility complex (MHC) region on chromosome 6 seem to be linked to EIMs in IBD. In CD, extraintestinal co-morbidities are more commonly observed in patients with HLA-A2, -DR1 and DQw5, whereas in ulcerative colitis, the genotypes HLA-DRB1*0103, B*27 and B*58 are linked with EIMs involving the joints, skin and eyes[8-10]. Fifty to eighty percent of IBD patients with ankylosing spondylitis are

HLA*B27 positive[11]. Furthermore, in UC the haplotype HLA B8/DR3 is associated with primary sclerosing cholangitis (PSC) and may also be linked to other autoimmune diseases (e.g. celiac disease, autoimmune hepatitis, myasthenia gravis)[12]. Interestingly, the NOD2 gene in CD seems to be associated not only with ileal disease in CD but also with sacroileitis[13].

In the following, an overview of the involvement of the different organ systems in IBD will concentrate on frequency, clinical presentation and therapeutic implications. In some cases, a differentiation of extraintestinal *manifestations* and extraintestinal *complications* will be given. Besides the classical extraintestinal manifestations, such as skin, joints, eyes and the hepatobiliary system, special attention will be given to the rarer, more recently described involvements, such as thromboembolic events, osteopenia and osteoporosis, pulmonary involvement and affection of the central nervous system.

## MUSCULOSCELETAL MANIFESTATIONS

Joint manifestations are the most common EIMs in IBD and occur in approximately 20%-30% of patients[3,14]. Males and females are equally affected. Symptoms may range from arthralgia only to acute arthritis with painful swollen joints. Both peripheral arthritis and axial arthritis can occur.

### *Peripheral arthritis*

Peripheral arthritis in IBD is quite distinct from specific forms of arthritis since there is little or no joint destruction, and tests for rheumatoid factor, antinuclear antibody and LE factor are negative. The prevalence of all forms of peripheral arthritis is reported to be between 5%-10% in UC and 10%-20% in CD, respectively[6,15], not considering asymptomatic patients under medical treatment. There are two types of peripheral arthritis in IBD[15] that should be distinguished from unspecific myalgia or arthralgia:

Type 1 (pauciarticular) arthritis affects less than five large joints (predominantly of the lower limbs) and the swelling is acute and often self-limiting. Type I arthritis is related to disease activity of the underlying bowel disease. The mean duration is 5 weeks; some 25%-40% of patients will have recurring arthritis. Type 2 (polyarticular) arthritis is a symmetrical polyarthritis, frequently involving five or more of the small joints (e.g. knuckle joints). Its course is independent of disease activity and may last for several months.

The etiology of peripheral arthritis in IBD is thought to be a combination of genetic predisposition and exposition to luminal (bacterial) bowel contents. Type 1 IBD arthritis is associated with HLA-DRB1*0103, HLA-B*27 and HLA-B*35, whereas type 2 IBD arthritis is associated with HLA-B*44 and MHC class I chain-like gene A, which is a non classical HLA gene located near the HLA-B on chromosome 6[8,10,16]. The site of intestinal inflammation is of particular interest concerning the pathogenesis of joint inflammation since CD patients with colonic involvement are at higher risk of developing arthritis than those with isolated small bowel disease. Furthermore the incidence of new joint complications significantly decreases after ileocecal resection (even when corrected for the time spent in remission after surgery), suggesting that bacterial overgrowth proximal to the ileocecal valve plays an important role in the pathogenesis of extraintestinal joint inflammation[6].

The diagnosis of peripheral arthritis in IBD is made clinically since radiographic findings do not show erosions or deformities. In persisting disease, a positive rheumatic factor should be excluded. In acute swelling, septic arthritis, fistulating arthritis or gout may be excluded by joint aspiration.

**Treatment:** Type 1 arthritis is related to disease activity and therefore therapy of the underlying IBD is the treatment of choice. Especially in patients with relapsing arthritis (HLA-DRB*0103), 5-ASA treatment should be switched to sulfasalazine, thereby taking advantage of the antiarthritic effect of sulfapyridine to minimize the risk of relapse. In addition, symptomatic treatment is often sufficient. For analgetic therapy, NSAID's and COX-2 selective inhibitors should be avoided, if possible, due to their potential to activate the underlying IBD[17]. In severe cases, symptoms relief can be achieved by intra-articular steroid injection. Type 2 IBD arthritis generally requires long-term treatment. In persisting disease, sulfasalazine should be initiated at an initial dose of 2 × 500 mg per day, increasing the daily dose by 1000 mg every two weeks towards the maximum dose of 3 × 1500 mg or until symptoms improve. If not effective despite 12 wk of continuing treatment, immunosuppression with methotrexate (7.5 mg po once weekly) should be started. The dose can be increased by 2.5 mg steps in monthly intervals up to the maximum dose of 25 mg per week. Concomitant folic acid (5 mg po 24 h following methotrexate) is recommended to reduce side effects. Systemic corticosteroids may be necessary to control symptoms.

### *Axial arthropathies*

The axial arthropathies are not associated with disease activity of IBD. Spondylitis occurs in 1%-26% of patients with IBD and males are more often affected than females. Both progressive ankylosing spondylitis and sacroiliitis (sometimes asymptomatic) may occur. Plain radiographs of the sacroiliac joints show uni- or bilateral sclerosis and/or erosions. The diagnostic gold standard is magnetic resonance imaging (MRI) with a high sensitivity in detecting sacroiliitis even in the absence of symptoms.

### *Ankylosing spondylitis*

Ankylosing spondylitis (AS) affects the vertebral column by progressive ankylosis of the vertebral facet joints and the sacroiliac joint (Figure 1). The prevalence of AS in IBD (1%-6%) is higher than in the general population (0.25%-1%)[18]. In contrast, the association with HLA*B27 is considerably weaker than in idiopathic AS with only 50%-80% of IBD patients being positive for HLA B*27 compared to 94% in the general population[11]. Again, bacteria and gut inflammation seem to play an important role in the pathogenesis of AS.

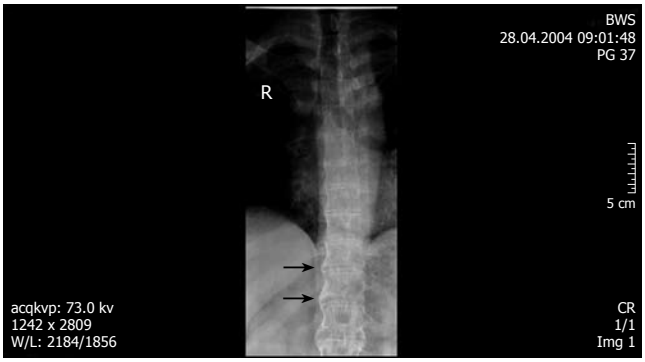


**Figure 1** X-ray thoracic spine demonstrating ankylosing spondylitis (Bechterew's disease) with syndesmophytes/bamboo spine in a patient with CD.

Interestingly, ileocolonoscopy in patients with idiopathic spondylarthropathies reveals ileal inflammation in more than two thirds of patients[19].

The clinical course of AS in IBD is similar to idiopathic AS, and disease progression leads to increasing immobility of the spine resulting in ankylosis (bamboo spine). Secondary to reduced chest expansion, poor lung expansion with fibrosis and dilatation of the aortic root can occur. AS is associated with peripheral arthritis in about 30% of patients and with uveitis in 25% of patients.

**Treatment:** There is no causative treatment and therefore physical therapy is of particular importance to maintain mobility of the spine. In the absence of active IBD, NSAID are the drugs of choice, otherwise acetaminophen or tramadol are preferred. Steroid injection (MRI-guided) into the sacroiliac joint may be an option in patients with severe low back pain[20]. Sulfasalazine may be used, but is more effective in associated peripheral arthritis. The first line immunosuppressant in patients with AS and IBD is methotrexate. Anti-TNF-strategies should be reserved for severe cases. Experience is limited to small case series, but improvement of both spondylarthropathy and active bowel disease has been reported in CD with infliximab[21-23]. Etanercept is effective in spondylarthropathies[24,25] but the efficacy in CD has not yet been demonstrated.

### *Isolated sacroiliitis*

It may occur in patients with IBD but most patients are asymptomatic and the disease is non-progressive. Prevalence depends on the radiological method used and varies from 18% in plain radiographs and 32% in CT imaging to 52% in radioisotope scintigraphy. Isolated sacroiliitis seems not to be associated with HLA*B27[6]. Asymptomatic HLA*B27 negative patients with normal spinal mobility do not require specific treatment.

### *Osteoporosis*

Patients with IBD have an increased risk of developing osteoporosis, associated with fragility fractures and morbidity. The overall prevalence of osteoporosis in IBD is approximately 15% but is more prevalent with older age; the overall relative risk of fractures is 40% greater when compared to the general population[26,27]. Vertebral fractures often occur spontaneously or after minimal trauma and it is estimated that only one-third of vertebral fractures come to clinical attention[28]. X-ray images of the spine most commonly show wedge or compression deformities. A variety of studies have demonstrated both decreased bone mineral density (BMD) in patients with IBD[29-38], and increased rates of bone loss when followed longitudinally[34,39,40] in comparison to healthy controls. The current Gold standard for measuring bone mass is dual-energy X-ray absorptiometry (DEXA)[41].

The pathogenesis of osteoporosis in IBD is multifactorial. Important pathogenetic factors in IBD include the cumulative steroid dose, hypogonadism induced by IBD (absence of menstrual period in women), malabsorption of calcium and vitamin D, low body mass index and disease activity/elevated inflammatory cytokines[42]. Other risk factors are previous fragility fracture, a positive family history, concomitant liver/endocrine disease (hyperthyroidism, hyperparathyroidism), immobilization and life style risk factors (smoking, excessive alcohol intake, physical inactivity). The multifactorial pathogenesis of bone loss in IBD makes it difficult to assess the importance of each single contributing factor. The results of a study from Norway indicate that disease activity and corticosteroid therapy are the most important factors involved in bone loss in CD patients[43]. However, it remains unclear whether the bone loss is related to the disease or to its treatment.

Biochemical markers of bone turnover (e.g., osteocalcin, bone specific alkaline phosphatase, carboxyterminal propeptide procollagen type 1, urinary deoxypyridinoline, pyridinoline, carboxytelopeptide of type I collagen, N-telopeptide cross-linked type I collagen) do not correlate sufficiently with current BMD for routine use[30,33,35,44-46] and should be confined to research studies[26].

**Treatment:** Few IBD patients are receiving optimal bone-sparing therapy, highlighting the importance of increasing awareness of osteoprosis in managing these patients[38]. Preventing bone loss should begin with an attempt to limit corticosteroid-induced bone loss. This can be done by minimizing the corticosteroid dosage, substituting with budesonide when appropriate[47,48], administering other steroid-sparing immunomodulators once corticosteroid dependence becomes evident, or by prescribing additional agents that enhance bone health. The administration of calcium and vitamin D[49] appears to maintain or enhance bone mass[26]. Bisphosphonates are of unclear additional benefit to the majority of patients who are at low fracture risk. In a small trial in Denmark, one year of daily alendronate treatment p.o. improved BMD in the spine[50]. Bisphosphonates (etidronate, risedronate, alendronate) are effective in preventing bone loss in steroid treated patients, but only few patients with IBD have been included in these trials[51-54]. Nasal or s.c. calcitonin can be considered as an alternative treatment approach when bisphosphonates are contraindicated or poorly tolerated. Testosterone replacement should be considered in hypogonadal men[26], estrogen replacement in postmenopausal women[55].

### *Osteomalacia*

Osteomalacia is a rare complication of IBD, most likely occurring in patients with severe CD and multiple

intestinal resections[56], resulting from prolonged and severe vitamin D deficiency. Though both osteoporosis and osteomalacia result in low BMD, apart from elevated bone alkaline phosphatase levels, osteomalacia can only be distinguished from osteoporosis by bone biopsy.

### *Joint complications in IBD*

Complications involving the joints should always be considered and have to be distinguished from sterile joint inflammation, since steroid treatment can cause osteonecrosis (avascular necrosis of the bone). Patients on immunosuppressive therapy are at increased risk of septic arthritis. In CD, fistulization may cause bacterial infection of the iliosacral joint. Rarely a psoas abscess can cause septic hip arthritis.

## MUCOCUTANEOUS MANIFESTATIONS

Erythema nodosum, pyoderma gangraenosum and oral ulceration are the most common cutaneous manifestations in IBD and are usually related to disease activity but sometimes may take an independent course. All patients presenting with IBD should be examined for cutaneous manifestations.

### *Erythema nodosum*

It is the most common skin manifestation in IBD affecting up to 15% of CD patients, with a female predominance[1,6,14]. Erythema nodosum (EN) affects the subcutaneous fat (septal panniculitis), causing tender erythematous nodules usually located on the shins (Figure 2). EN normally heals without ulceration and the prognosis is good. The clinical picture is typical and biopsy rarely is required for diagnosis. The etiology of EN is unknown, however there is a genetic association with a distinct HLA region on chromosome 6 (HLA-B)[9]. EN characteristically parallels intestinal disease activity. There is an association with other EIMs such as arthritis and uveitis[14,15]. Treatment of the underlying bowel disease usually results in improving EN lesions and at least 25% of EN will heal spontaneously. There is no specific treatment for EN, but symptomatic therapy should comprise pain medication and oral steroids may be given in severe cases. Immunosuppressive treatment is not necessary. In the absence of active bowel disease, other causes of EN, such as sarcoidosis or post-streptococcal infection, should be taken into consideration.

### *Pyoderma gangrenosum (PG)*

Pyoderma gangrenosum occurs in 0.5%-2% of both patients with UC and CD and may take a course independent of disease activity (Figure 3). Conversely, 36%-50% of patients with PG suffer from IBD. PG appears as a tender erythematous papule evolving into a livid pustule with central necrosis and subsequent ulceration, occurring in single or multiple lesions. The ulcer often has a irregular outline and is sharply demarcated with a heaped-up mushy violaceous border, surrounded by a erythematous zone (Figure 1). Often minor trauma, needle stitches or biopsy can induce new PG lesions

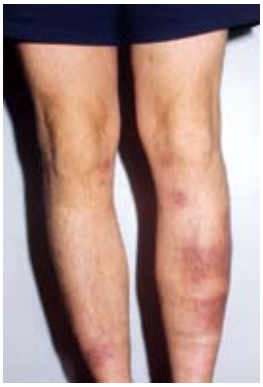

**Figure 2** Erythema nodosum in a patient with CD.

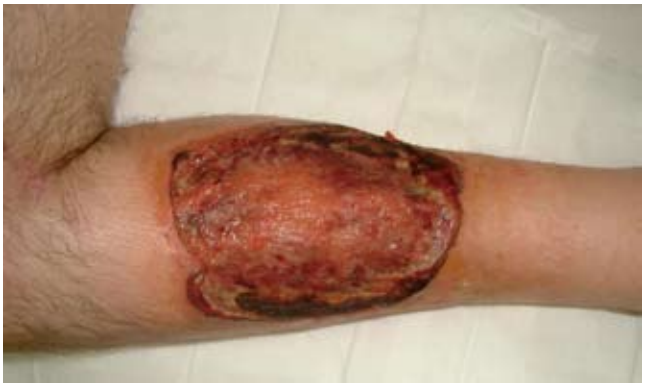

**Figure 3** Pyoderma gangrenosum in a patient with CD.

(pathergy phenomenon). PG lesions have a predilection for the lower limbs but may occur in any area of the skin, sometimes even as peristomal ulcers[57]. The diagnosis of PG is made clinically; nevertheless skin biopsy of the border of the ulcer may be performed to rule out vasculitis or infection. There are no pathognomonic histologic features, generally revealing only diffuse neutrophilic infiltration and dermolysis.

PG is the most severe skin manifestation in IBD. PG is painful and often persisting despite adequate therapy. Without treatment, PG can last for years and ulcers may spread. Therefore, aggressive and early treatment is required. Local wound care consists of dressings, mild débridement of necrotic material and eschar (continuous wet saline compressions, topical enzymatic ointment, hydrocolloid dressings similar to common ulcer treatment). Topical tacrolimus has also emerged as potentially useful therapy[58]. First line systemic treatment in PG is high dose prednisolone. Intravenous pulse therapy over three days is highly effective. Careful tapering should be started with clinical improvement. In steroid dependent or steroid refractory (no improvement within 5 d) cases, immunosuppressive therapy should be initiated. In mild cases a combination of steroids with dapsone has been successfully used with an initial dosage of dapsone 100 mg po/d, gradually increasing to 200-300 mg/d[59]. In more severe cases cyclosporine or tacrolimus are effective. Steroid dependent patients require immunosuppressive treatment with azathioprine/6-mercaptopurine. A variety of other treatments (thalidomide, topical cromoglycate,

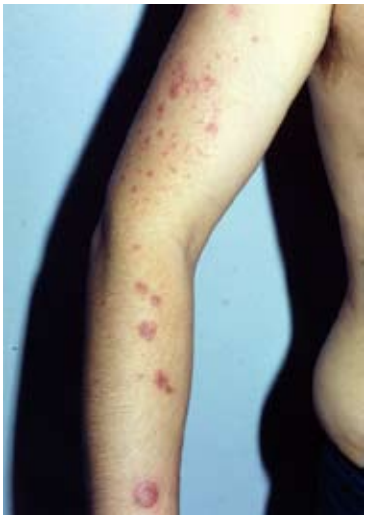

**Figure 4** Sweet syndrome: papulosquamous exanthema in a patient with UC.

clofazimine, plasmapheresis, granulocyte apheresis, hyperbaric oxygen) have been reported anecdotally. Surgical intervention should be avoided, if possible, since it may induce pathergy. In resistant cases infliximab at a dose of 5 mg/kg has been used successfully[59,60]. PG often takes a prolonged course, but in general will be controllable with medical therapy. About 35% of patients will experience relapsing PG.

### *Oral ulcerations*

Oral aphthous ulcers occur in at least 10% of patients with UC and 20%-30% with CD and rapidly resolve once remission is achieved. Stomatitis, as an adverse event of methotrexate therapy, should be taken into account.

### *Miscellaneous skin lesions*

Many other skin affections have been described in patients with IBD, such as Sweet's syndrome (neutrophilic dermatosis)[61] (Figure 4), leukocytoclastic vasculitis, psoriasis, epidermolysis bullosa acquisita, and cutaneous polyarteriitis nodosa. Since these diseases have been mainly reported as single case reports, they probably occur coincidentially rather than as a true EIM. Angular cheilitis in nearly 8% of patients with CD often is a sign of iron deficiency.

## EYE MANIFESTATIONS

Two to five percent of patients with IBD experience ocular manifestations[62]. The manifestations range from conjunctivitis to more significant inflammation, including iritis, episcleritis, scleritis and anterior uveitis. Mild cases of conjunctivitis may be diagnosed clinically, but in other cases early referral to an ophthalmologist is important for accurate diagnosis.

### *Episcleritis*

Episcleritis is less common in UC than in CD, presents as an infection of the ciliary vessels and an inflammation of the episcleral tissues and does not affect visual acuity. Inflammation episodes tend to occur in association with active bowel disease. Successful treatment consists of both topical corticosteroids and treatment of the underlying bowel disease. Scleritis affects deeper layers of the eye and can cause lasting damage if untreated.

### *Uveitis*

Uveitis is less common than episcleritis and occurs in 0.5%-3% of patients. It does not affect visual acuity unless it involves the posterior segment. Uveitis frequently presents bilaterally, is insidious in onset and chronic in duration. It is more common in females and may not parallel bowel disease activity. On slit-lamp examination uveitis presents as a perilimbic edema and "inflammatory flare" in the anterior chamber. Conjunctival vessel injection and corneal clouding may also be seen. An acute episode of uveitis can lead to permanent damage of the eye with iris atrophy, lens deposits or synechiae. More aggressive therapy may be necessary, especially when the posterior chamber is affected. Prompt diagnosis and therapy with topical and systemic steroids is crucial and sometimes intraocular injections of corticosteroids may be necessary. Steroid treatment should be continued for four weeks before tapering if uveitis is under control. Iridospasm is relieved by topical mydriatic eyedrops. Successful treatment for IBD-associated uveitis with infliximab was first described in one CD patient having a suitable extraintestinal constellation (uveitis and sacroileitis)[63]. In rheumatoid arthritis with severe refractory uveitis, infliximab was effective but with an unexpected high rate of side effects[64].

Ocular complications in IBD include keratopathy and night blindness resulting from malabsorption of vitamin A. Cataract development is a severe side effect of steroid therapy, therefore regular ophthalmologic examination should be considered. Rare eye manifestations are retinal vascular disease (central vein occlusion or vasculitis), peripheral corneal ulcers, corneal infiltrates and central serous chorioretinopathy with bullous retinal detachment.

## HEPATOBILIARY MANIFESTATIONS

### *Primary sclerosing cholangitis*

In UC, the main hepatic EIM is primary sclerosing cholangitis (PSC), a chronic inflammatory disease of the biliary tree, occurring in approximately 3% of all patients (Figure 5). The diagnosis is made by endoscopic or magnetic resonance cholangiography, showing beading, irregularity, and stricturing of intrahepatic and extrahepatic ducts. Histology ranges from obliterating concentric fibrosis of the bile ducts to chronic inflammatory infiltrates in the portal tracts resulting in interface hepatitis. Low titres of autoantibodies against smooth muscle, parietal cells, and nuclear antigens are common, and high titres of autoantibodies to neutrophils (p-ANCAs)[65], showing a perinuclear pattern of staining, have been described. The majority (70%) of patients have the HLA-DR3, B8 haplotype.

### *Treatment*

Ursodeoxycholic acid has been suggested to delay disease progression[66]. Although not avoiding the progression of liver disease, ursodeoxycholic acid has been demonstrated

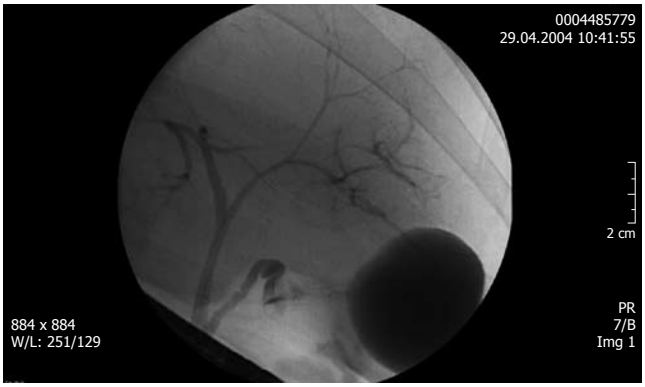


**Figure 5** ERC demonstrating primary sclerosing cholangitis in a patient with CD.

to exhibit chemopreventive effects and reduce the risk of colorectal dysplasia in ulcerative colitis[67,68]. This is of importance since patients with UC and concomitant PSC have a significantly higher risk of developing colorectal neoplasia compared with patients having UC only[69]. PSC is seen as a premalignant condition for the development of cholangiocarcinoma as well.

Early symptomatic treatment of cholestasis is mandatory to avoid septic complication and consists of endoscopic dilatation with or without stent insertion. PSC is slowly progressive and independent of the course of UC, developing the complications of portal hypertension and chronic liver failure. For end-stage liver disease, liver transplantation remains an option.

PSC may occur in 4% of patients with CD as well, usually in those with colonic disease. Inflammatory changes of the small ducts show a normal cholangiogram and pericholangitis on liver biopsy[70].

### *Hepatobiliary complications*

Gallstones are frequent in patients with CD, of which 25% develop gallstones mainly due to malabsorption of bile salts from the inflamed terminal ileum. Abnormalities of liver function (elevated serum aminotransferase and alkaline phosphatase) are common in patients with malnutrition, sepsis, and fatty liver due to severe attacks of IBD. These correlate with disease activity and return to normal once remission is achieved. Factors favouring fatty infiltration of the liver in severely ill patients are poor nutrition and often concomitant steroid therapy.

## PANCREATIC MANIFESTATIONS

There is an increased risk for acute as well as chronic pancreatitis in IBD[71-73] documented by a multitude of case reports and case series.

### *Acute pancreatitis*

Especially in cases of acute pancreatitis, it may be difficult to determine the true incidence of EIM[74]. Many drugs in IBD treatment have the potential to induce acute pancreatitis (e.g. salicylates, azathioprine and 6-mercaptopurine, rarely corticosteroids). Drug-induced pancreatitis typically occurs within the first weeks after commencing drug-therapy[75], the course is usually mild and resolves quickly after discontinuing the drug. Especially in CD regional inflammatory complications due to duodenal/papillary involvement or biliary complications should be considered. Nevertheless, after excluding extraintestinal complications, an increased risk for idiopathic pancreatitis remains in patients with IBD (1%-1.5%) that should be considered as true extraintestinal involvement.

Pancreatic autoantibodies have been found in up to 40% of CD patients[76]. In contrast to earlier reports[77] the prevalence seems to be increased in both CD and UC patients as well as in first-degree relatives[78]. The relevance for disease is still unclear, however. In a series with 64 antibody-positive CD patients the proportion suffering from exocrine insufficiency was 27% compared to 8% in antibody negative patients[79]. Further studies are needed to show whether these antibodies, apart from their diagnostic relevance for IBD, actually play a role in pathogenesis and whether they can help to identify patients at risk of developing an EIM involving the pancreas.

### *Chronic pancreatitis*

Endoscopic retrograde pancreatography (ERP) is still the most sensitive and specific test for chronic pancreatitis. There are several reports on intrapancreatic duct changes in patients with IBD[80,81]. Pancreatic function has been investigated as well. In the study of Heikius, the exocrine function was found to be decreased in only 4% of patients using the secretin test. These results are consistent with two other studies that diagnosed exocrine insufficiency in a minority of IBD patients only[73,82]. In all studies, chronic alcohol ingestion as a potential cause for chronic pancreatitis had been excluded. The pathogenesis of chronic pancreatitis in patients with IBD remains unclear. Since there has only been one documented case of pancreatic granuloma in Crohn's disease[83], pancreatitis has been considered to be caused by circulating inflammatory mediators rather than directly-involved pancreatic tissue. As discussed above, autoantibodies against pancreatic tissue may play a role in the development of exocrine insufficiency.

## THROMBOEMBOLIC EVENTS

Patients with IBD are at increased risk of developing thromboembolic complications. The incidence of deep vein thrombosis and pulmonary embolism is increased and is a major cause of mortality in IBD. Venous thrombembolism is more common than arterial embolism. Conventional risk factors, such as prolonged immobilization, hospitalization, sepsis, surgery or invasive procedures, contribute to this increased risk especially in active or complicated IBD. In severe disease, thrombocytosis and increased concentrations of many clotting factors that behave as acute phase proteins, lead to a procoagulatory status. The majority of IBD patients with thromboembolic events have active disease[84]. However, a recent study demonstrated nicely that IBD, as such, is a risk factor for thromboembolic events. Comparing patients with IBD, rheumatoid arthritis and celiac disease to age and sex-matched controls, they found that patients

with IBD have a 3.6-fold increased risk of experiencing thromboembolic events in contrast to the other chronic inflammatory diseases that have no increased risk[85].

There is no consistent evidence that inherited thrombophilia is associated with IBD. The main established genetic risk factors have not been found to be increased in IBD[86-88]. In more than half of patients with thrombosis, no predisposing factor is evident[86]. Therefore, screening for these risk factors seems to be justified only in the case of a personal or a family history of thromboembolism. Hyperhomocysteinemia, more common in IBD patients than in controls, is associated with an increased risk of thrombembolism as well. To date it is controversial if polymorphisms in the methylenetetrahydrofolate reductase (MTHFR) gene leading to hyperhomocysteinemia are more frequent in IBD patients compared to healthy controls[87,89,90]. Lack of Vitamin B6, B12 or folate or the use of folate-inhibiting drugs (methotrexate, sulfasalzine) can contribute to acquired hyperhomocysteinemia.

Corticosteroid treatment may cause a hypercoagulable state with increased risk of thrombosis, but systemic data pointing towards a significant association is missing. In general, all hospitalized patients with IBD should receive low-dose heparin for thromboprophylaxis unless there is severe bleeding.

## MANIFESTATIONS OF THE KIDNEYS AND THE URINARY TRACT

### *Glomerulonephritis*

There are several reports on glomerulonephritis in patients with IBD. Many kinds of histology and clinical course, ranging from minimal change nephropathy to rapidly progressive glomerulonephritis, have been described[91-94]. A true extraintestinal manifestation is difficult to prove, however, and the combination of glomerulonephritis and IBD may well be coincidental.

### *Interstitial nephritis and tubular proteinuria*

Again, it is difficult to make a distinction between EIM and side effects of medication. Single cases of interstitial nephritis have been reported in IBD. At least one case of granulomatous interstitial nephritis has been attributed to underlying CD[95], thus interstitial nephritis may represent a EIM of IBD. Medication, especially therapy with aminosalicylates, may cause interstitial nephritis as well. However, surveillance data from France[96] and the UK[97] report a low incidence of renal impairment under salicylate therapy. An additional, large epidemiologic study recently demonstrated that renal impairment is attributed to severity of the underlying IBD rather than to 5-ASA treatment[98]. This is in accordance with the fact that the frequently observed tubular proteinuria in IBD is related to disease activity and not dependent on 5-ASA treatment[99-101]. The clinical relevance of this proteinuria remains to be determined.

### *Renal and urinary complications*

In patients with CD, uric acid and oxalate stones are common. Due to fat malabsorption luminal calcium is bound to free fatty acids. Lack of free calcium results in increased oxalate absorption, hyperoxaluria and formation of renal oxalate stones. Hyperoxaluria may cause chronic tubulointerstitial nephritis as well. Prevention treatment of oxalate stones consists of low oxalate diet and supplementation of oral calcium (1-2 g/d). The risk for uric acid stones is increased with volume depletion (e.g. due to diarrhoea, ileostomy) and a hypermetabolic state in critically ill patients. Prevention of uric acid stone recurrence consists of hydration and oral urinary alkalization.

A rare complication is renal amyloidosis (AA-Amyloidosis) due to chronic inflammation; it mainly occurs in CD and is rarer in UC. Systemic AA amyloidosis is caused by extracellular deposition of fragments of the circulating acute-phase-plasma protein (SAA). Usually the mean duration between onset of the underlying chronic disease and the occurrence of amyloidosis is at least 15 years. The diagnosis is confirmed by detection of amyloid (Congo red staining) deposits in tissue (rectum biopsy, abdominal fat aspiration). Progression of amyloidosis can be stopped by controlling inflammation. Additional genitourinary complications may be caused by (enterovesical) fistula formation, perivesical abscesses, cystitis and obstructive uropathy.

## PULMONARY MANIFESTATIONS

Various pulmonary manifestations have been reported in IBD including small and large airway dysfunction as well as obstructive and interstitial lung disorders. Case reports do not show a uniform picture of disease. Various entities including bronchial hyperreagibility, bronchitis and bronchiectasis, inflammatory tracheal stenosis, interstitial pneumonitis as well as bronchiolitis obliterans organizing pneumonia (BOOP) have been described. The respiratory symptoms usually follow the onset of the IBD. Sometimes pulmonary disease, especially serositis, correlates with IBD activity. Parenchymal lung disease often develops independently of disease activity. Interestingly, colectomy seems to be a risk factor for developing pulmonary symptoms with a frequent postoperative onset[102,103].

Recently, a large population-based Canadian study reported airway disease as the most common concomitant chronic disease in patients with CD and second most common in patients with UC[3]. Patients with CD and with UC were more likely, 30%-40% and 50%-70% respectively, than controls to have asthma[3]. The high frequency of pulmonary involvement in IBD probably reflects the commonality of bronchus-associated and gut-associated lymphoid tissue[104]. Subclinical lung involvement is much more common than apparent respiratory symptoms, reported in as many as 30%-60% of patients with CD. Bronchoalveolar lavage reveals alveolitis in as much as 50% of CD patients without any clinical respiratory symptoms[105] and abnormal pulmonary function tests have been reported in 42% of IBD patients without respiratory symptoms compared to only 3% in controls[106]. Interestingly, these changes persist during remission.

Persistent airway inflammation can result in airway narrowing, dependent on the localization, resulting in

tracheal stenosis, bronchiectasis or bronchiolitis obliterans. In fact, the majority of patients with IBD-related respiratory manifestations present with chronic bronchitis or bronchiectasis[102]. Chest X-rays are often unspecific; high resolution chest CT scanning may demonstrate bronchial wall thickening, dilated airways, branched opacities due to mucoid impaction[107]. In lung biopsy nonspecific inflammation, small airway fibrosis and sometimes granulomatous bronchiolitis have been reported[108,109]. The most common interstitial lung disease associated with IBD is bronchiolitis obliterans organizing pneumonia (BOOP)[102]. Chest X-ray reveals patchy focal opacities or diffuse infiltrates, while CT scanning often demonstrates pleural-based opacities and air bronchograms. Typical granulomatous interstitial lung disease in Crohn's disease is rare and may mimic sarcoidosis with the occurrence of noncaseating granulomas and elevated CD4/CD8 ratios in bronchoalveolar lavage fluid[110-112]. Apart from airway inflammation and interstitial lung disease other pulmonary manifestations of IBD include serositis[113]. Serositis occurs as exsudative pleural effusion pericarditis, pleuropericarditis and myocarditis[107].

### Complications

Pulmonary parenchymal involvement may be related to IBD, but also may be induced by drugs (e.g. mesalazine, sulfasalazine, methotrexate). Many cases of drug-induced pulmonary complications in IBD patients have been reported. Salicylates of all kinds can induce different types of interstitial lung disease, such as BOOP and granulomatous lung disease. The pulmonary toxicity of 5-aminosalicylic acid (5-ASA) is less common than with sulfasalazine, but pulmonary infiltrates, sometimes eosinophilia or BOOP may develop as well[102,114,115]. Pulmonary infiltrates with eosinophilia (PIE syndrome) can occur with or without the use of sulfasalazine or mesalamine[116]. Chest X-rays often show peripheral infiltrates typical of chronic eosinophilic pneumonia and laboratory values reveal eosinophilia. Methotrexate treatment may cause serious hypersensitivity pneumonitis or pulmonary fibrosis. Chest X-rays show diffuse alveolar or interstitial infiltrates.

Especially if patients receive combination immunosuppression, the possibility of opportunistic pulmonary infections should be taken into account.

### Treatment

It is unclear whether asymptomatic patients should receive therapy at all. However, it should be born in mind that IBD patients carry an increased mortality risk due to pulmonary disease[117-119]. Depending on the type of pulmonary complication, inhaled or systemic steroid therapy may be effective. In various forms of airway inflammation, inhalatory steroid therapy (e.g. beclomethasone up to 1200 μg/d) is generally effective, with large airway inflammation being more responsive than bronchiolitis. Patients with bronchiectasis are less likely to respond to inhaled steroids and may require oral steroids. In severe airway inflammation with upper airway obstruction, such as subglottic involvement, intravenous steroids may be required. Interstitial lung disease usually requires systemic steroids; e.g. prednisolone 0.5-1.0 mg/kg per day for a longer period of time, usually months, depending on the clinical course.

## CARDIAC MANIFESTATIONS

Pericarditis or perimyokarditis with or without an effusion have been described in a few case reports on patients with active IBD. Again, it may be difficult to rule out drug toxicity since salicylates are potential culprits. However, in the Canadian population-based study, both patients with CD and UC were at an increased risk of developing pericarditis in comparison to healthy controls[3]. Age-adjusted prevalence ratios were 3.07 in CD and 3.33 in UC, respectively, although the numbers were small[120].

## NEUROLOGICAL MANIFESTATIONS

The association of autoimmune neurological disorders and IBD has long been hypothesized, especially for multiple sclerosis[121-125]. Furthermore there are some sporadic reports of neuropathies in IBD such as optic neuritis[126], peripheral neuropathies[127] and (subclinical) sensorineural hearing loss[128,129]. MRI studies have shown clinical nonapparent cerebral white matter lesions in patients with IBD[130]. A recent retrospective cross-sectional study evaluated the risk of patients with IBD to develop either multiple sclerosis, optic neuritis or demyelinating disease in the pre-anti-TNF-era[131]. The authors found a small but significant association of IBD with these demyelinating diseases (OR 1.67). The risk was not found to be related to the use of steroids and/or immunosuppressive therapy with thiopurines. These results are of special interest in the light of recent reports on new unexpected adverse events of biological agents.

Recently, alarming reports on the development of progressive leucencephalopathia under treatment with natalizumab, an antibody directed against α4-integrin, have resulted in stopping all ongoing trials with this substance[132,133]. Similar observations exist on the anti-TNFα-therapeutic agents infliximab, etanercept and adalimumab[134-136] and have led to additional safety warnings concerning the use of these drugs. To date, patient numbers are too small to draw final conclusions but these agents may well accelerate the preexisting risk for demyelinating disorders in IBD patients.

Metronidazole, often used in fistulizing CD, can cause polyneuropathia if given as long term treatment. Nutritional deficiencies (Vitamin B12) should always be taken into consideration and looked for especially in long-lasting and severe disease.

## SUMMARY

In conclusion, IBD are systemic disorders and not restricted to the intestine. Recent reports on the involvement of new organ systems emphasize that almost every site of the body can be affected by the inflammatory process. The possibility of contributing

**Table 1 Extraintestinal symptoms in inflammatory bowel disease**

| Extraintestinal manifestations | Extraintestinal complications |
|---|---|
| **Musculosceletal diseases** | |
| Peripheral Arthritis | Drug-induced osteoporosis and osteonecrosis |
| Ankylosing spondylitis | Bacterial infection of joints (fistulization, immunosuppression) |
| Isolated sacroiliitis | Septic arthritis |
| Metastatic Crohn's disease | |
| **Mucocutaneous diseases** | |
| Erythema nodosum | Anal fissures |
| Pyoderma gangraenosum | Fistulas |
| Aphthous stomatitis/oral ulceration | |
| Psoriasis? | Acrodermatitis enteropathica (Zinc deficiency) |
| Epidermolysis bullosa acquisita? | Purpura (Vitamin C and K deficiency) |
| Sweet Syndrome? | Glossitis (Vitamin B or Zinc deficiency) |
| Erythema exsudativum multiforme? | Hair loss (protein deficiency)<br>Brittle nail (protein deficiency)<br>Perlèche (Iron deficiency)<br>Candidiasis (Zinc deficiency, immunosuppression)<br>Mucositis/stomatitis (methotrexate)<br>Drug-induced rash, allergic exanthema<br>Moon-face, acne, stretch marks, skin atrophy (steroid treatment) |
| **Ocular diseases** | |
| Anterior Uveitis | Night blindness Vitamin A deficiency |
| Conjunctivitis | Kerathopathy Vitamin A deficiency |
| Iritis<br>Skleritis/Episcleritis | Opportunistic infections (immunosuppressants) |
| **Hepatobiliary diseases** | |
| Primary sclerosing cholangitis | Gall stones in Crohn' disease |
| Granulomatous Crohn's hepatitis | Fatty liver |
| Autoimmune chronic active hepatitis? | |
| Biliary cirrhosis? | |
| **Pancreatic diseases** | |
| Acute pancreatitis | Drug-induced pancreatitis (5-ASA, azathioprine) |
| Chronic pancreatitis, exocrine insufficiency | Biliary pancreatitis in Crohn's disease |
| | Duodenal involvement in Crohn's disease |
| **Blood and vascular diseases** | |
| Thrombembolic events | Anemia (Iron-, folate-, vitamin B12-deficiency) |
| Autoimmune hemolytic anemia? | Thrombocytosis, leucocytosis |
| Thrombocytopenic purpura (Moschcowitz Syndrome)? | Hypercoagulation: venous thrombosis, thrombembolism |
| **Renal diseases** | |
| Tubular proteinuria | Nephrolithiasis (oxalate stones, uric acid stones) |
| Glomerulonephritis? | Local affection involving the urethro-genital system |
| | Acute interstitial nephritis (drug related SASP, 5-ASA) |
| Interstitial nephritis? | Drug-induced renal insufficiency (5-ASA, SASP, cyclosporine) |
| | Renal amyloidosis |
| **Bronchopulmonary diseases** | |
| Chronic bronchitis/bronchiolitis/bronchiectasis | Drug-induced hypersensitivity pneumonitis |
| Acute laryngotracheitis/tracheal stenosis | Drug related pulmonary fibrosis (methotrexate) |
| Bronchiolitis obliterans organizing pneumonia | Drug-induced pleuritis |
| Pleuritis/serositis | Opportunistic infections (immunosuppression) |
| **Cardiac diseases** | |
| Pericarditis | Drug-induced pericarditis (5-ASA) |
| Myocarditis?/perimyocarditis | |
| **Neurological diseases** | |
| Demyelinating diseases including multiple sclerosis | Peripheral neuropathy (Vitamin B12 deficiency) |
| Optic neuritis, sensorial hearing loss | Drug-induced leucencephalopathia (natalizumab, infliximab) |
| Myasthenia gravis? | Drug-induced polyneuropathia (metronidazole) |

disease complications, especially through the side effects of treatment, should always be born in mind. The distinction between disease and treatment side effects can be extremely difficult and may sometimes be impossible. Recent reports on multiple organ involvement, including the central nervous system, point out the importance of an increased awareness for these potential problems. In addition, these data provide additional warning concerning the use of the new biological treatment strategies. These agents have to be prescribed with care since long term experience with toxicity is limited.

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*World J Gastroenterol* 2006 August 14; 12(30): 4832-4835
***World Journal of Gastroenterology*** ISSN 1007-9327


*GASTRIC CANCER*

# Clinical profile of gastric cancer in Khuzestan, southwest of Iran

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## Abstract

**AIM:** To analyze the characteristics of epidemiological, clinical and survival patterns among patients with carcinoma of the stomach.

**METHODS:** We retrospectively studied the characteristics of 186 gastric adenocarcinoma patients at Ahwaz Jundishapur University Hospitals (AJSUH) from September 1, 1996 to September 1, 2002. All the patients had histopathologically-confirmed malignancy. Demographic variables, family history of gastric cancer (GC), clinicopathologic characteristics and treatment-related variables were analyzed. Univariate analysis was performed with the log-rank test and multivariate analysis with Cox regression. $P < 0.05$ was considered statistically significant.

**RESULTS:** Male to female ratio was 2.6:1. The mean age was 60.6 years and 14% of the patients were younger than 40 years. Adenocarcinoma, gastric lymphoma, and gastric metastasis were found in 94.5%, 2.3%, and 3% patients, respectively. There was an average of 6-mo delay between the initial symptoms and the diagnosis. Among adenocarcinoma groups, intestinal type was the commonest (55.9%) and the distal third was the most common localization (88.4%). One hundred and thirty-four patients (72.1%) were males. Thirty-one patients (17%) had a family history of GC. Surgery was performed in 90% of patients (non-curative).

**CONCLUSION:** The epidemiological features of GC in south Iran mimic those in high-risk areas. There is a higher frequency of GC in young patients at our institution. Patients are detected and treated after a relatively long delay. Most patients present in advanced stages, which favors a poor overall survival. Family history of GC has a significant problem in our area. Studying the etiology of this cancer in south Iran and earlier diagnosis and subsequent better cares are recommended.



**Key words:** Gastric cancer; Epidemiological features; Khuzestan; Southwest of Iran

Eskandar H, Hossein SSM, Rahim M, Jalal H, Mehrdad A, Rajabi T. Clinical profile of gastric cancer in Khuzestan, southwest of Iran. *World J Gastroenterol* 2006; 12(30): 4832-4835

http://www.wjgnet.com/1007-9327/12/4832.asp

## INTRODUCTION

Gastric cancer (GC) remains the third most common malignancy in the world[1]. The pattern and incidence of GC vary widely in different parts of the world. Costa Rica and Japan have the first and second highest rates in the world with a rate of 77.5 and 50.5/100 000 persons, respectively[2,3]. Gastric cancer is the most common malignancy in Iran and its incidence is particularly high in the Ardabil Province in the northwest of the country. In this province, the age standard incidence rate is 49.1 and 25.4/100 000 persons per year in males and females, respectively. The cause of the high incidence of GC in this geographical region is unknown[4,5].

The epidemiology of GC has been widely studied in the Western world as well as in Japan[6-9]. However, only few scattered reports from the developing world have been published[10-14]. There is a lack of good descriptive data on GC from the Middle East countries, where both cancer registration and prevalence of risk factors are relatively unknown. Because of the decreasing trend taking place in the Western world as a result of possibly socio-economic development and its consequences, it is important to gain an insight into what is happening in other parts of the world such as in the Middle East. This prompted us to report the epidemiological and clinicopathological features of gastric malignancy in Khuzestan, southwest of Iran in comparison to other countries whenever possible. This

could assist in the better understanding of the important risk factors, which contribute to the development of GC. This also gives a clue about whether or not screening programs are needed in our region.

## MATERIALS AND METHODS

We retrospectively analyzed the clinicopathologic characteristics of 186 gastric adenocarcinoma patients who were admitted to or operated upon at Ahwaz Jundishapur University Hospitals (AJSUH) from September 1, 1996 to September 1, 2002. Age, sex, method of operation, size of lesion, location of cancer in the stomach and stage were analyzed in patients, retrospectively. Histologically confirmed primary gastric malignancy was found in 186 patients, including 177 patients with adenocarcinoma, 5 patients with primary gastric lymphoma (PGL), and 3 patients with malignant gastric metastasis. All available endoscopy reports were reviewed. At the entry, clinical symptoms, demographic data and medical history were recorded and gastroscopy was performed to establish the endoscopic diagnosis and status of *H pylori* infection. During the examination of biopsy specimens from the stomach, silver or modified Giemsa staining and histological examinations were performed to establish the diagnosis and status of *H pylori* infection.

Patients and/or family members were contacted. Gastric adenocarcinoma was classified into intestinal type (IT), diffuse type (DT) or mixed type according to the histological criteria of Lauren[15]. Tumor staging in each patient was based on clinical information, preoperative radiological investigations, operative findings and pathological examination. The staging was made in accordance with the TNM system[16].

Clinicopathologic data were compared using the $\chi^2$ and Fisher's exact tests. $P < 0.05$ was considered statistically significant.

## RESULTS

During the study period, 186 patients with GC were identified, 127 (68.3%) patients were males with a male to female ratio of 2.6:1. The peak incidence was in the age group of 60-69 years (40%), followed by the age group of 50-59 years (16%). Approximately 14% of the patients were younger than 40 years and 6% of the patients were younger than 30 years. The mean age for the whole group was 63 years (range 21-91 years). Table 1 shows the age distribution in the patient groups.

Features of the patients are summarized in Table 2. There was an average of 6-mo delay (range 1-13 mo) between the initial symptoms and diagnosis.

Carcinomas were located most frequently in the lower third of the stomach, accounting for 88.5% (165/186) of all patients. Table 3 shows the distribution of various histological types of gastric adenocarcinoma according to the sites that were affected. One hundred (53.5%) and eighty-six (46.5%) patients lived in urban and rural areas, respectively. The histopathology of gastric biopsy showed *H pylori*-associated chronic active gastritis in 166 (88.9%) patients.

**Table 1 Age distribution in patient groups**

| Age (yr) group | Male patients (*n*) | % | Female patients (*n*) | % |
|---|---|---|---|---|
| 1-10 | 0 | 0 | 0 | 0 |
| 10-19 | 0 | 0 | 0 | 0 |
| 20-29 | 4 | 2.15 | 7 | 3.76 |
| 30-39 | 15 | 8 | 7 | 3.76 |
| 40-49 | 11 | 6 | 6 | 3.2 |
| 50-59 | 20 | 11 | 10 | 5.4 |
| 60-69 | 50 | 27 | 24 | 12.9 |
| 70-79 | 19 | 10 | 6 | 3.2 |
| ⩾ 80 | 7 | 3.7 | 0 | 0 |
| Total | 126 | 67.9 | 60 | 32 |

**Table 2 Features of the studied patients**

| Signs and symptoms | Patients (*n*) | % |
|---|---|---|
| Abdominal pain | 93 | 50 |
| Weight loss | 23 | 12 |
| Dyspepsia | 91 | 48.8 |
| Nausea, vomiting | 74 | 40 |
| Abdominal mass | 4 | 2 |
| Anorexia | 182 | 97.7 |
| Dysphagia | 30 | 16 |
| Gastrointestinal bleeding | 26 | 14 |
| Ascites | 28 | 15 |
| Constipation | 5 | 2.5 |

**Table 3 Distribution of gastric adenocarcinomas according to their site *n* (%)**

| Histopathological type | GEJ and gastric cardia | Gastric corpus and antrum | Total |
|---|---|---|---|
| Intestinal type adenocarcinoma | 24 (12.9) | 80 (43.1) | 104 |
| Diffuse type adenocarcinoma | 14 (7.5) | 60 (32.3) | 74 |
| Gastric lymphoma and metastasis | 0 (0) | 8 (4.3) | 8 |
| Total | 38 (16.7) | 148 (79.9) | 186 |

GOJ: Gastro-esophageal junction.

According to TNM staging, the proportions of stages ⅠA, Ⅰ, Ⅱ + Ⅲ, and Ⅳ in the studied groups were 0% (0/186), 28% (53/186), and 71% (133/186), respectively (Table 4).

## DISCUSSION

Gastric cancer is the most prevalent malignancy in Iran. If GC is diagnosed at an early stage, patients can have a highly favorable prognosis and avoid extended surgery, which may produce complications, especially in the elderly people. However, the symptoms of GC are non-specific and vague, when symptomatic patients experience epigastric pain and discomfort and definitive symptoms such as weight loss or obstructive symptoms and metastases that often impede curative radical resection. Additionally, the results of GC treatment do not differ

**Table 4 TNM stage and methods of operation in patient groups**

| Stage (TNM) and methods of operation | Patients (*n*) | % |
|---|---|---|
| Ⅰ | 0 | 10 |
| Ⅱ | 20 | 10.8 |
| Ⅲ | 33 | 17.5 |
| Ⅳ | 133 | 71 |
| **Curability** | | |
| Curative resection | 15 | 8 |
| Palliative resection | 95 | 51 |
| No resection | 76 | 41 |
| **Type of resection** | | |
| Total and subtotal gastrectomy | 70 | 37.6 |
| Distal gastrectomy | 14 | 7.5 |
| Other resections and palliative operation | 26 | 14 |

markedly from the past results though there are improved surgical techniques and adjuvant treatments. Researchers have shown that the prognosis of GC has not changed in the past 20 years[9,17]. The only method that is likely to improve the survival rates is early detection of GC. Our patients tended to present late as evidenced by the fact that there was a long interval between the onset of symptoms and presentation. There was an average of 6-mo delay (range 1-13 mo) between the initial symptoms and the diagnosis.

This is not due to the insufficient current endoscopic services, but due to the fact that many people in our area who have dyspeptic symptoms visit non-specialist physicians who either prescribe medications for long term treatment or use drugs in order to ameliorate the pain. Subsequently, some of these patients whose cause of dyspepsia is cancer present with late stage GC or one of its complications. In addition, the elderly people usually fail to make use of the available medical services. However, general practitioners should be more liberal in referring patients for endoscopy and resist the temptation to treat dyspeptic patients with anti-ulcer therapy without endoscopy, especially in elderly people and in patients with alarming signs. Open access endoscopy, greater efforts in patients' education and improvement of the diagnostic technical skills may improve the early detection of GC.

According to TNM staging, the proportions of stages ⅠA, Ⅰ, Ⅱ+Ⅲ, and Ⅳ in the studied groups were 0% (0/186), 28% (53/186), and 71% (133/186), respectively. Approximately more than two-thirds of the patients were diagnosed with advanced GC.

These results re-emphasize that GC symptoms are non-specific and need an early screening examination. A public screening system for gastric cancer has not yet been introduced in Iran and in our area.

Gastric cancer is the most common malignancy in Iran and its incidence is particularly high in the Ardabil Province in the northwest of the country[12,13,18]. In this province, the age standard incidence rate is 49.1 and 25.4/100 000 persons per year in males and females, respectively.

The cause of the high incidence of gastric cancer in our country is unknown.

The following two factors may play a role. First, the rapid change in dietary habits constitutes a risk factor. Vitamin C-rich fresh vegetables and fruits, starch and natural unprocessed wheat products are the major constituents of Iranian food. However, canned food, hot spices, pickles and animal proteins are now dominating the Iranian menu.

Fermentation of foods involves the production of nitrosamine. This compound has been implicated as a risk factor for GC in many studies and frequent consumption of fermented food may be a risk factor for GC in our region because our people use such compounds most often.

However, to further investigate the association between fermentation and GC, more comprehensive and detailed data are required. Salt has been indicated as a risk factor for GC in many previous studies. Since the use of salt and fermentation in our regional food preparations is strongly inter-related, we are unable to clearly separate the independent effects of the two variables.

It is known that the environmental risk factors for GC are dietary in origin[19,20].

In our region the incidence of *H pylori* infection (> 80%) is high and there is a substantial incidence of reflux disease. In addition, 30% people smoke, < 5% people drink alcohol and 60% people have a body mass index > 25[21].

Most resections are done with palliative intent. The low rate of gastrectomy with "curative" intent could be explained by the high proportion of patients with advanced GC at presentation.

Patterns of GC in our area are similar to those reported from high-risk regions worldwide[22]. In our study, the male to female ratio was 2.6:1, the peak incidence was in the age group of 60-69 years (40%), followed by the age group of 50-59 years (16%). Approximately 14% of the patients were younger than 40 years and 6% of the patients were younger than 30 years. The mean age of the whole group was 63 years (range 21-91 years). Among our study groups intestinal type (IT) adenocarcinoma was the commonest histological subtype (56%).

IT adenocarcinoma was more common than DT adenoma (1.65: 1) and distal location was more frequent than the proximal one (4.77:1) (Table 1).

In Western countries, PGL and metastasis are represented only in 2%-5% of gastric malignancies[23]. It was 4.3% in our series, which was lower than 9% from neighboring Iraq[24], and 14%-22% from Saudi Arabia[11,25]. During the past three decades the site of PGL in the Middle East has changed. Small intestinal involvement has become less common and gastric involvement more frequent. This varying pattern seems to be environmental in origin. The ratio of PGL to gastric adenocarcinoma among our patients was 0.045, which is similar to the western series[26].

In conclusion, several symptoms of GC are non-specific. The majority of identified gastric adenocarcinoma patients are symptomatic, and have a lesser chance of being cured by operation and a lower survival rate. The patients with dyspeptic symptoms and alarming signs should be referred to earlier diagnostic endoscopy. Improvements in diet and food storage and control of *H pylori* infection, by indirect means such as improving the

general sanitary conditions or by direct interventions such as eradication are likely to offer great potentials for the prevention of GC in this area.

Although this study has highlighted the pertinent epide-miological and clinicopathological features of gastric malignancy in Khuzestan Province in Iran, further studies are needed to evaluate the environmental risk factors, incidence, the treatment outcomes and survival rate.

## ACKNOWLEDGMENTS

The authors would like to thank the medical and nursing staff of Emam and Golestan hospitals for their help in conducting this study and the faculty of Medicine of Jundishapur University, for their encouragement and support.

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**S- Editor** Wang J **L- Editor** Wang XL **E- Editor** Liu WF

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*World J Gastroenterol* 2006 August 14; 12(30): 4836-4842
***World Journal of Gastroenterology*** ISSN 1007-9327


*VIRAL HEPATITIS*

# HepG2 cells support viral replication and gene expression of hepatitis C virus genotype 4 *in vitro*

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Received: 2005-10-18 Accepted: 2006-01-24

## Abstract

**AIM:** To establish a cell culture system with long-term replication of hepatitis C virus (HCV) genome and expression of viral antigens *in vitro*.

**METHODS:** HepG2 cell line was tested for its susceptibility to HCV by incubation with a serum from a patient with chronic hepatitis C. Cells and supernatant were harvested at various time points during the culture. Culture supernatant was tested for its ability to infect naïve cells. The presence of minus (antisense) RNA strand, and the detection of core and E1 antigens in cells were examined by RT-PCR and immunological techniques (flow cytometry and Western blot) respectively.

**RESULTS:** The intracellular HCV RNA was first detected on d 3 after infection and then could be consistently detected in both cells and supernatant over a period of at least three months. The fresh cells could be infected with supernatant from cultured infected cells. Flow cytometric analysis showed surface and intracellular HCV antigen expression using in house made polyclonal antibodies (anti-core, and anti-E1). Western blot analysis showed the expression of a cluster of immunogenic peptides at molecular weights extended between 31 and 45 kDa in an one month old culture of infected cells whereas this cluster was undetectable in uninfected HepG2 cells.

**CONCLUSION:** HepG2 cell line is not only susceptible to HCV infection but also supports its replication *in vitro*. Expression of HCV structural proteins can be detected in infected HepG2 cells. These cells are also capable of shedding viral particles into culture media which in turn become infectious to uninfected cells.



**Key words:** Hepatitis C virus; *In vitro* propagation; Genomic replication; Gene expression; HepG2 cells

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http://www.wjgnet.com/1007-9327/12/4836.asp

## INTRODUCTION

The lack of an efficient cell culture system or a readily available small animal model has hampered the development of therapies for hepatitis C virus (HCV) infection. The chimpanzee is the only animal that is susceptible to hepatitis viral infections, but its endangered status and financial considerations limit its widespread use in viral hepatitis research. Despite these difficulties, recent introduction of heterologous cDNA expression systems[1] and subgenomic replicons[2] have allowed researchers to study various aspects of the viral life cycle and examine novel antiviral therapies. Also, among the surrogate animal models that have been developed are mouse liver repopulated with human hepatocytes and transgenic mice expressing hepatitis antigens[3-5]. For reasons that are not evident, infection of primary hepatocytes and established cell lines with hepatitis viruses have not only produced poor viral replication and low viral yields but have also suffered from poor reproducibility[6]. The entry of virus into a cell, followed by productive viral replication, depends on both viral and host cell proteins. Only differentiated cells may express the latter. Thus, studies of HCV and HBV infectivity initially used

primary hepatocytes from humans or chimpanzees. One group infects human fetal hepatocytes with HCV-infected serum[7]. The viral replication is quite low and detectable only by RT-PCR amplification. Using this technique, another group showed an increase in the number of HCV+ strands by d 5, indicating that these hepatocytes support viral replication. Similarly, yet another group showed that adult primary human hepatocytes could be infected with HCV in culture conditions that support long-term cultures of hepatocytes for at least 4 mo[8]. Under these culture conditions, viral positive-strand RNA was first detectable by PCR after 10 d of infection, and the viral RNA titer increased in culture media during a 3-mo culture. This group also demonstrated *de novo* synthesis of negative-strand viral RNA. Culture supernatants from HCV-infected hepatocytes could transmit infection to naive hepatocytes, indicating the production of infectious viral particles. However, the efficiency of viral infection is unpredictable and does not correlate with viral RNA titers. Addition of polyethylene glycol to the primary hepatocyte cultures maintained in the presence of 20 g/L dimethylsulfoxide markedly increases the infection of HBV[9] but not HCV[10]. HCV is lymphotropic, and peripheral blood mononuclear cell cultures support HCV replication[11]. However, the level of viral replication is very low[12]. Because primary hepatocytes are difficult to grow in cultures, some researchers have attempted to infect immortalized hepatocytes and hepatoma cell lines. Ikeda and colleagues[13,14] used PH5CH, a nontumorigenic, immortalized human hepatocyte cell line, to assess the infectivity of HCV positive sera. There was an increase in the HCV sense -strand RNA during the first 12 d of culture, and the viral RNA remained detectable for at least 30 d after infection. Nucleotide sequence determination of the HCV genome in the hypervariable region 1 showed that there is a shift toward the limited HVR-1 population, indicating strong selection for HCV variants during the infection[13]. Furthermore, IFNγ inhibits the viral replication in these cells[14]. Recently, Guha *et al*[5] reported that *in vitro* cell culture models can at best demonstrate the infectivity of the virus but are not suitable to study viral life cycle because of the very low levels of viral replication. These systems could be used in evaluating drugs for antiviral activity or inhibition of HCV infection. Also, Horscroft *et al*[15] have summarized the recent development of HCV replicon cell culture system and its use in anti-HCV drug discovery. In the present study, we tested the susceptibility of HepG2 cell line to HCV and established an infection cell model that could support HCV long-term replication *in vitro*. The presence of both sense- and antisense-RNA strands and expression of viral core and envelope proteins in infected cells as well as the ability of these cells to exocytose infectious viral particles into culture media suggests that the current cellular model allows study of HCV life cycle.

## MATERIALS AND METHODS

### HEPG2 cell culture

Caucasian male *Homo sapiens* (human) hepatocellular carcinoma cell line (HepG2; ATCC, HB-8065, Manassas, USA) was used to establish the *in vitro* HCV replication. HepG2 culturing and infection were carried out according to the protocols described by Seipp *et al*[10]. HepG2 cells were maintained in 75 $cm^2$ culture flasks (greiner bio-one GmbH, Germany) containing Dulbecco's modified Eagle's medium (DMEM) supplemented with 4.5 g/L glucose and 10 g/L L-glutamine (Bio Whittaker, a Combrex Company, Belgium) containing 100 mL/L fetal calf serum (FCS; Biochrome KG Berlin Germany), 10 g/L antibiotics (penicillin/streptomycin; Biochrome KG, Berlin, Germany) and 1 g/L antimycotic (fungisone 250 mg/L; Gibco-BRL life Technologies, Grand Island, New Y). After adding all supplements the medium is called complete. The culture medium was renewed by a fresh medium every 3 d, and cells were subcultured (6-10 d).

In summary the medium was discarded, the adherent cell layer was shortly treated with trypsin-EDTA (2.5 g/L; Sigma, Deisenhofen, Germany) to remove the left traces of trypsin inhibitors from the FCS contained in the medium. After discarding, 1.0 mL of fresh trypsin-EDTA was added onto the cells and flasks were kept either at room temperature or at 37℃ (5-15 min) to observe the dettachement of cells from the flask wall. To avoid extended proteolytic effect of trypsin on the dettached cells complete medium was added to inhibit the enzyme activity. Cells were spun down at 400 g for 2 min, resuspended in 1 mL of complete medium, the exact count of cells was recorded in 50 μL aliqout after mixed with equal volume of trypan blue (5 g/L; BiochromKG, Berlin, Germany.) using a hemocytometer (Right Line; Sigma, Deisenhofen, Germany). A total of $3 \times 10^6$ cells were suspended in 10 mL complete medium and incubated at 37℃ in 5% $CO_2$.

### Viral inoculation and sample collection

Cells were grown for 48 h to semi-confluence in complete medium, washed twice with FCS -free medium, then inoculated with a serum sample (500 μL sense and 500 μL FCS-free DMEM/$3 \times 10^6$ cells) obtained from HCV infected patients (RT-PCR and antibody positives). The HCV genotype in the used sera was previously characterized as genotype 4 based on the method described by Ohno *et al*[16]. The viral load in the used serum was quantitated by real time PCR and the average copy number was $290 \times 10^6$ copies/L. After 90 min, DMEM containing FCS was added to make the overall serum contents 100 mL/L in a final volume of 8 mL including the volume of human serum used for infection as mentioned above (0.04483 copies/cell). Cells were maintained overnight at 37℃ in 5% $CO_2$. On the next day, adherent cells were washed three times with culture medium to get rid of the remaining infection serum and incubation was continued in complete medium containing 100 mL/L FCS with regular medium changes. The viral infection in HepG2 cells throughout the culture duration was assessed qualitatively by sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting of viral antigens, RT-PCR amplification of sense and antisense strands and quantitatively by real time PCR.

### Flow cytometric analysis of intracellular staining of HCV core antigen in infected HEPG2 cells

The intracellular staining of HCV core antigen in infected HepG2 cells was quantified by using a fluorescence activated cell sorting (FACS) based assay. Intracellular staining labeling was performed by direct immuno-fluorescence. HepG2 cells (collected after addition of trypsin) were centrifuged and supernatants were removed. Cell pellets were washed 4 times with PBS. For intracellular staining, cells were incubated with 4% paraformaldehyde for 10 min and 0.1% Triton X-100 in Tris buffer (pH 7.4) for 6 min. After washed with PBS, cells were incubated with FITC-labeled $F(ab)_2$ portion of HCV core antibody (at 1:2000 dilution) for 30 min at 4℃. Cells were washed with PBS containing 1% normal goat serum and suspended in 500 μL and analyzed by flow cytometry (FACS Calibure, BD). Mean fluorescence intensity was determined using Cell Quest software (Becton Dickinson).

### Flow cytometric analysis of labeled E1 antigen on surface of infected HEPG2 cells

The surface staining of HCV E1 antigen in infected HepG2 cells was quantified by using a fluorescence activated cell sorting (FACS) based assay. Surface labeling was performed by direct immuno-fluorescence. HepG2 cells collected after trypsinization were centrifuged and supernatants were removed. Cell pellets were washed 4 times with PBS. Cells were incubated with FITC labeled HCV E1 antibody (at 1:1500 dilution) for 30 min at 4℃. Cells were washed 3 times with PBS containing 10 mL/L normal goat serum and suspended in 500 μLPBS and analyzed with flow cytometry (FACS Calibure, BD). Mean fluorescence intensity was determined using Cell Quest software (Becton Dickinson).

### Western blot analysis of HCV antigens in HEPG2 cells

Uninfected and infected HepG2 cell lysates were subjected to sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE)[17] through 40 g/L stacking and 160 g/L resolving gels in 0.75 mm-thick vertical slab gels. Cell lysate samples were diluted at 1:25 in PBS, mixed with the sample buffer (0.125 mol/L Tris base, 40 g/L SDS, 2% glycerol, 100 g/L mercaptoethanol, and 1 g/L bromophenol blue as a tracking dye) and immediately boiled for three min. A mixture of reference proteins was run in parallel. Gels were then stained with Coomassie blue. Western blotting was performed as follows: resolved samples separated by SDS-PAGE were electro-transferred onto nitrocellulose membranes (0.45 mm pore size). On the next day, membranes were cut into individual strips each of 0.3 mm width. Strips were washed 3 times with PBS-3 g/L T each for 5 min and blocked against non specific binding at room temperature for 1 h in PBS-3 g/L T-10 g/L bovine serum albumin (BSA). Strips were washed 3 times as above and incubated with diluted first antibody (infected human serum at 1:100, or anti-core/envelope rabbit antibodies at 1:500) in PBS-3 g/L T at room temperature for 2 h. After washed 3 times, strips were incubated with diluted peroxidase-labeled second antibodies (anti-human IgG/IgM mixture at 1:5000 in PBS-3 g/L T for previously treated strips with human sera or anti-rabbit IgG at 1:1000 in PBS-3 g/L T for those treated with rabbit anti-core/envelope antisera. Both antibodies were from Jakson Immuno Research Laboratories; Dianova, Hamburg, Germany) for 2 h at room temperature. Visualization of immune complexes on the nitrocellulose membrane was done by developing the strips with 0.01 mol/L PBS (pH 7.4) containing 50 mg diaminobenzedine (Sigma; Deisenhofen, Germany) and 100 μL of 30 mL/L hydrogen peroxide.

### Isolation and extraction of RNA from serum and HEPG2 cells

Isolation and extraction of RNA from serum and HEPG2 cells were performed as reported in our previous study[18]. Briefly, cells were precipitated and washed in the same buffer to remove adherent viral particles before lysis in 4 mol/L guanidinium isothiocyanate containing 25 mmol/L sodium citrate and 0.5% sarcosyl and 0.1 mol/L b-mercaptoethanol. Cellular RNA was extracted using the single-step method described originally by Chomczynski and Sacchi[19].

### PCR of genomic RNA strands of HCV

Reverse transcription-nested PCR was carried out according to Lohr *et al*[20] with few modifications. Retrotranscription was performed in 25 mL reaction mixture containing 20 units of AMV reverse transcriptase (Clonetech, USA) with either 400 ng of total PBMC RNA or 3 mL of purified RNA from serum samples (equivalent to 30 mL serum) as template, 40 units of RNAsin (Clontech, USA), a final concentration of 0.2 mmol/L from each dNTP (Promega, Madison, WI, USA) and 50 pmol of the reverse primer P1 (for sense strand) or 50 pmol of the forward primer P2 (for anti-sense strand). The reaction was incubated at 42℃ for 60 min. and denatured at 98℃ for 10 min. Amplification of the highly conserved 5' -UTR sequences was done using two rounds of PCR with 2 pairs of nested primers. The first round amplification was done in 50 mL reaction containing 50 pmol from each of P2 forward primers and P3 reverse primers, 0.2 mmol/L from each dNTP, 10 μL from RT reaction mixture as template and 2 units of Taq DNA polymerase (Promega, USA) in 1 × buffer supplied with the enzyme. The thermal cycling protocol was as follows: 1 min at 94℃, 1 min at 55℃ and 1 min at 72℃ for 30 cycles. The second round amplification was done as the first round, except for use of the nested reverse primer P4 and forward primer P5 at 50 pmol each. A fragment of 172 bp was identified in positive samples. Primer sequences are as follows: P1: 5' ggtgcacggtctacgagacctc 3'; P2: 5' aactactgtcttcacgcagaa 3'; P3: 5' tgctcatggtgcacggtcta 3'; P4: 5' actcggctagcagtctcgcg 3'; P5: 5' gtgcagcctccaggaccc 3'. To control false detection of negative-strand HCV RNA and known variations in PCR efficiency[21,22], specific control assays and rigorous standardization of the reaction were employed as previously described[20]. These specific control assays were cDNA synthesis without RNA templates to exclude product contamination, cDNA synthesis without RTase to exclude Taq polymerase RTase activity, cDNA synthesis and PCR step done with only the reverse or forward

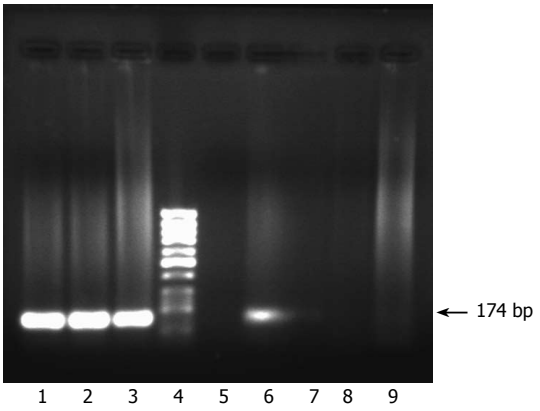


**Figure 1** Establishment of an *in vitro* infection experiment of HepG2 cells with serum of a HCV genotype 4-infected patient and monitoring success of infection by nested RT-PCR amplification of viral sense and minus strands. The patient was confirmed to be infected with HCV as demonstrated by nested RT-PCR amplification of viral positive strand in the serum (lane 1), and both viral positive and negative strands (lane 2 and lane 3 respectively) in the peripheral blood mononuclear cells. After infection of the cells with the patient sera (see the detailed method in the materials and methods), cells were carefully washed and nested RT-PCR was carried out on the last wash to make sure that the culture medium contained no more viral RNA and results showed no amplified products (lane 5). At this stage we were sure that any detection of viral RNA within the cells could reflect successful viral adsorption and penetration. Three days after infection, RNA was extracted from both cells and their supernatant and nested RT-PCR for detection of both viral strands was carried out and results showed the presence of the sense strand in the cells (lane 6) but not in the supernatant (lane 7). The antisense strand was neither present in the cells nor in the supernatant (lanes 8, 9). Lane 4 is molecular weight standard DNA marker (Ø-X-174/HaeIII; Q-BIOgene, Germany).

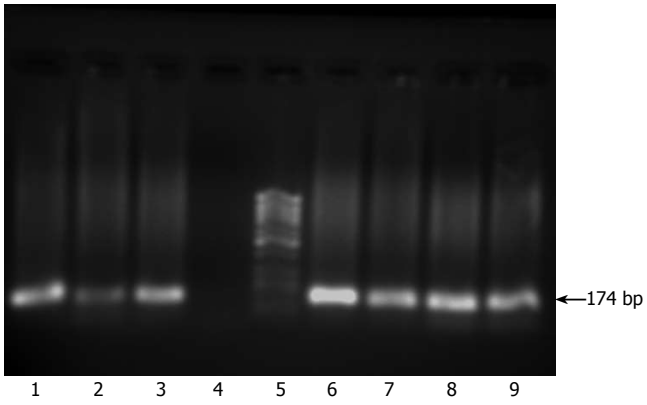


**Figure 2** Monitoring of active viral replication at regular time intervals post infection of HepG2 cells. RNA was extracted from infected cells and infectious supernatants and their passages at 1, 2 and 4 wk post infection and nested RT-PCR was carried out for detection of both viral strands. Results showed the presence of both viral strands in RNA extracted from cells 1 wk post infection (lanes 1, 2) but only the sense strand was detectable in the supernatant at this time point (lane 3) while the antisense strand was absent (lane 4). At 2 and 4 wk post infection, both viral strands were detectable in both cells and supernatant. Lanes 6-9 show the presence of both sense and antisense strands in both cells and supernatant at 4 wk post infection. Lane 5 shows molecular weight standard DNA marker (Ø-X-174/Hae Ⅲ; Q-BIOgene, Germany).

primer to confirm no contamination from mixed primers. These controls were consistently negative. In addition, cDNA synthesis was carried out using only one primer followed by heat inactivation of RTase activity at 95℃ for 1 h, in an attempt to diminish false detection of negative-strand prior to the addition of the second primer.

## RESULTS

### *Establishment of HCV HEPG2 cells in culture*

Success of infection was monitored by nested RT-PCR amplification of viral sense and antisense (minus) strands (Figure 1). To confirm the infection of HCV in a patient with chronic active hepatitis whose serum was used in infection of HepG2 cells, nested RT-PCR amplification of viral sense strand in the serum (lane 1) and both viral sense and antisense strands (lane 2 and lane 3 respectively) in peripheral blood mononuclear cells were demonstrated. The viral load was quantified in patient's serum as $2.9 \times 10^5$ using real time PCR method (results not shown). After infection of the cells with the patient's sera, cells were carefully washed and nested RT-PCR was carried out on the last wash to make sure that the cell wash contained no more viral RNA (lane 5). At this stage we were sure that any detection of viral RNA within the cells could reflect successful viral binding and entry. Three days after infection, RNA was extracted from cells and culture media. Nested RT-PCR was carried out for detection of both viral strands. Results shown in Figure 1 displayed the presence of sense RNA strand in the cells (lane 6) but not in the culture media (lane 7). The negative strand was neither present in the cells nor in the culture media (lanes 8, 9).

### *Monitoring of active viral replication at regular time intervals after infection of HEPG2 cells*

RNA was extracted from infected cells and infectious supernatants and their passages at 1, 2 and 4 wk post infection and nested RT-PCR was carried out for detection of both viral strands (Figure 2). Results showed the presence of both viral strands in RNA extracted from cells 1 wk post infection (lanes 1, 2) but only the positive strand was detectable in the supernatant at this time point (lane 3) while the negative strand was undetectable (lane 4). At 2 and 4 wk post infection, both viral strands were detectable in both cells and supernatant. Lanes 6-9 show the presence of both sense and antisense strands in both cells and supernatants 4 wk post infection. Results at 2 wk were not demonstrated.

### *Monitoring infection of HEPG2 cells using culture medium from primary infected cells by nested RT-PCR*

After incubation of HepG2 cells with infectious medium presumably containing exocytosed viral particles from primary infected cells, *de novo* infected cells were carefully washed to get rid of any viral traces and the last wash was checked for presence of viral RNA using nested RT-PCR which produced no amplified products (Figure 3, lane 1). RNA was extracted from infected cells as well as their culture media at 3 d, 1 wk, 2 wk and 4 wk post co-incubation with the infectious medium and subjected to nested RT-PCR to check the presence of either or both viral strands (Figure 3). After 3 d the cells contained only sense viral strand (lane 2) while the anti-sense strand was undetectable (lane 3). The supernatant contained neither strand (lanes 4, 5). After 1 wk post infection,

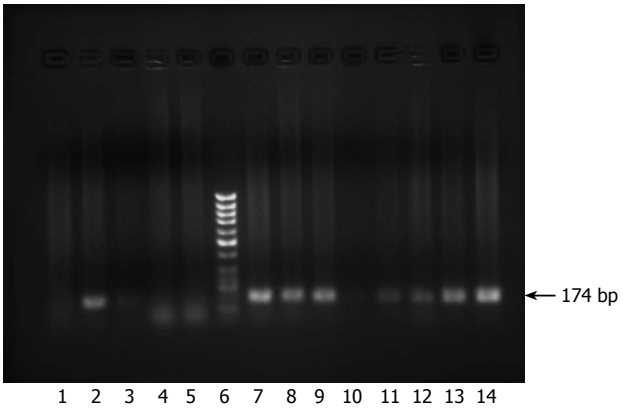


**Figure 3** Monitoring infection of HepG2 cells using culture medium from primary infected cells by nested RT-PCR. After incubation of HepG2 cells with infectious medium presumably containing exocytosed virions from primary infected cells, cells were carefully washed to get rid of any viral traces and the last wash was subjected to RNA extraction followed by nested RT-PCR. Results showed no amplification of positive strand products (lane 1). RNA was extracted from infected cells and supernatants at 3 d, 1, 2 and 4 wk post co-incubation with the infectious supernatant and subjected to nested RT-PCR to check for the presence of each viral strand. At 3 d the cells contained only the positive viral strand (lane 2) but the negative strand was undetectable (lane 3). However, the supernatants contained none of the strands, only primers used could be seen (lanes 4, 5). Lane 6 is molecular weight standard DNA marker (Ø-X-174/HaeⅢ; Q-BIOgene, Germany). At 1 wk post infection, both sense and antisense strands were detectable in the RNA extracted from the infected cells (lanes 7, 8), whereas the supernatant contained only the sense strand (lane 9) but not the antisense strand (lane 10). At 2 and 4 wk, RNA extracted from infected cells as well as the supernatants contained both positive and negative strands. Results of amplification for both positive and negative strands for cellular RNA are shown (2 wk, lanes 11, 12) and (4 wk, lanes 13, 14).

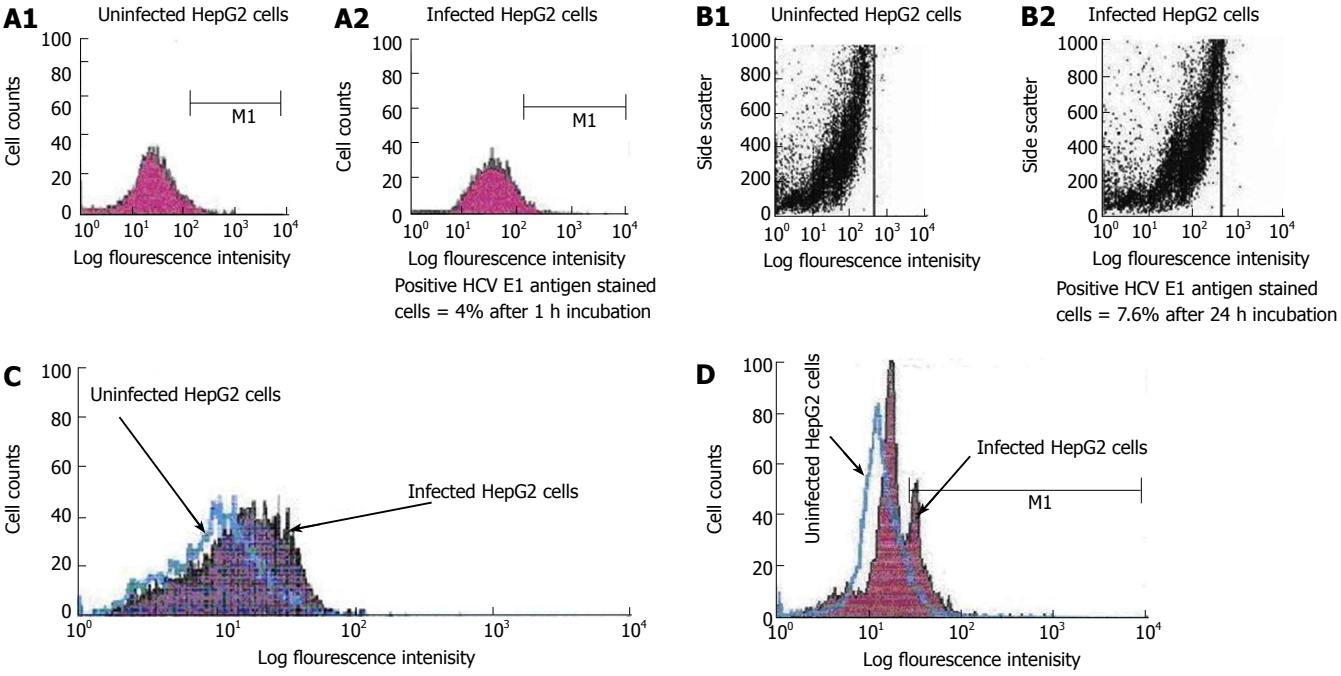


**Figure 4** **A**: Single parameter histogram for flow cytometric analysis of surface staining of HCV E1 gene expression on the infected HepG2 cells after one hour incubation. HepG2 cells were incubated with PBS (**A1**) (uninfected) or with HCV positive serum (**A2**) (infected) for 1 h incubation. Cells were harvested and stained with FITIC labeled HCV anti-E1 antibody as described in materials and methods; **B**: Dot histogram for flow cytometric analysis of surface staining of HCV E1 gene expression on the infected HepG2 cells after 24 h incubation. HepG2 cells were incubated with PBS (**B1**) (uninfected) or with HCV positive serum (**B2**) (infected) for 24 h incubation. Cells were harvested and stained with FITIC labeled HCV anti-E1 antibody; **C**: Overlap histogram for flow cytometric analysis of surface staining of HCV E1 gene expression on the infected HepG2 cells after one week incubation. HepG2 cells were incubated with PBS (uninfected) or with HCV positive serum (infected) for one week incubation. Cells were harvested and stained with FITIC labeled HCV anti-E1 antibody; **D**: Overlap histogram for flow cytometric analysis of intracellular staining of HCV core gene expression in the infected HepG2 cells after 3 d incubation. HepG2 cells were incubated with PBS (uninfected) or with HCV positive serum (infected) for 3 d incubation. Cells were harvested and stained with FITIC labeled HCV anti-core antibody Labeled cells were analyzed with flow cytometry (FACS Calibure, Becton Dickinson).

both sense and antisense viral strands were detectable in infected cells (lanes 7, 8), whereas the supernatant contained only the sense strand (lane 9) while the antisense strand was undetectable (lane 10). After 2 and 4 wk post infection, RNA extracted from infected cells as well as their supernatants contained both positive and negative strands. Results of amplification of both positive and negative strands from cellular RNA at 2 and 4 wk are presented in lanes 11-14. Results of the nested RT-PCR on the supernatant at the same time points were not demonstrated since they were identical to those obtained from the cells. However, culture supernatant from infected HepG2 cells was used to infect naïve (uninfected) cultured HepG2 cells and we found that these HepG2 cells were infected as detected by RT-PCR (Data not shown).

### *Flow cytometric analysis of surface and intracellular staining of HCV antigen expression in infected HEPG2 cells*

Flow cytometric analysis showed that HCV core and E1 antigens were detected on surface and inside of the infected HepG2 cells. Figures 4A-C show the percentage of anti E1 positive staining on the surface of HepG2 cells after 1 h (4%) and 24 h (7.6%) and one week (12.5%) of incubation of HepG2 cells with positive HCV serum sample. Core protein was detectable in 5.7% of cells after

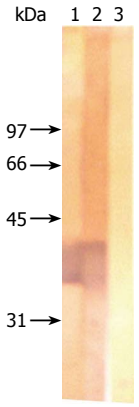


**Figure 5** Testing translation of viral E1 in supernatant and lysates of HepG2 cells infected with HCV from 1 mo culture by Western blot analysis. Supernatant (strip 1) and lysates (strip 2) of HepG2 cells infected with HCV were subjected to Western blot analysis, hybridization with the anti-E1 antibody clearly showed the expression of a cluster of immunogenic proteins at molecular weights localized between 31 and 45 kDa. This cluster was undetectable on the strip immobilized with uninfected HepG2 cell lysates (strip 3).

24 h, and increased to 13.5% of cells after 3 d. Figure 4D shows the intracellular staining of core antigen using F(ab)2 portion of the core antibody after infection of 3 d.

### *Western blot analysis of HCV viral antigen expression in infected HEPG2 cell lysates*

When supernatant (Figure 5, strip 1) and lysates (strip 2) of HepG2 cells infected with HCV were subjected to Western blot analysis, hybridization with the anti-E1 antibody could clearly show the expression of a cluster of immunogenic peptides at molecular weights extended between 31 kDa and 45 kDa over 1 mo period. This cluster was undetectable on the strip immobilized with uninfected HepG2 cell lysates (strip 3).

## DISCUSSION

Although knowledge of the molecular biology of HCV has progressed rapidly, our understanding of viral replication and pathogenicity is still hampered by the lack of reliable and efficient cell culture systems. To achieve a reliable *in vitro* system we need to obtain a biological status wherein viral-host interactions mimic exactly what happens naturally *in vivo*, since both viral and host factors make up together the overall outcome of the pathogenetic pathways. The reasons for using HepG2 cells in the current study include the great similarity in biosynthetic pathways between primary hepatocytes and HepG2 cells[23]. Also the later cells contain LDL and CD81 receptors which are known to mediate HCV entry into cells[24]. Validity of HepG2 cells in propagating HCV has been reported by other laboratories[25]. The viral component of the model has several alternative strategies. Subgenomic or genomic replicons have been used in elucidating the replicative machinery of the virus[26] but could not mimic the actual viral replication cycle and shedding of the virus to the culture medium. Despite the extremely robust *in vivo* replication rate of HCV using genomic replicons, efforts to propagate the virus in cell culture have been frustratingly unsuccessful[27]. Thus the viral replication but not the biologically relevant infectious viral particles can be demonstrated by such approach. In the present study we utilized infectious serum with native viral particles presumably containing the full length viral RNA genome in infecting HepG2 cells *in vitro*. The recent understanding of the HCV molecular biology demonstrates that both 5' and 3' untranslated regions of the viral RNA genome play a pivotal role in translation of viral proteins *via* interaction with cellular factors including eukaryotic initiation factor 3 eIF3[35], 40S ribosomal subunit[28] and poly pyrimidine tract binding (PTB)[29] protein. Besides, it has been shown that intra genetic viral interactions such as NS4a/NS5a are required for key pathways in HCV life cycle. In the current study, the use of infectious viral particles containing intact RNA genome could guarantee the presence of the necessary elements involved in translation of polyprotein precursor and viral replication. We have presented several lines of evidence that the cell model described herein maintains HCV life cycle. A minor fraction of cells (4%) had a detectable viral envelope on cell surface as early as one hour after incubation. This fraction steadily increased to 7.6% in 24 h and 12.5% after one week. E1 protein reached detectable levels by Western blotting analyses at both intracellular and extra cellular compartments after one month. *De novo* synthesis of RNA minus strand was detected inside HepG2 cells as early as one week post infection and appeared in the medium one week later. However, the detection of the replicative intermediate (antisense strand HCV RNA) is thought to be reasonable for assessment of HCV replication. Because detectable HCV structural proteins in cells after infection may represent the residue of the inoculated virus after releasing the viral genome to cytoplasm, it is necessary to demonstrate that HCV structural proteins detected in the infected cultures are newly synthesized rather than residuals of viral inoculum. Interestingly, the core protein was only detectable in 5.7% of cells after 24 h and increased to 13.5% of cells after 3 d, indicating that such observed increase in core expression reflects part of *de novo* synthesized structural viral proteins. The ability of culture medium to transmit viral particles to new cells later in one month culture with concomitant detection of core (results not shown) and envelope proteins as well as detection of sense and antisense RNA strands suggest that infected HepG2 cells reach a state of equilibrium after one month of infection. Other cellular models for HCV propagation can transmit viral particles to naïve cells[30]. We assume that this approach brings our *in vitro* system to become closer to native viral infection status occurring *in vivo*. The expression of different viral antigens agrees with the earlier reports that liver and blood cells from infected patients do support these expressions[11,31-35]. Our observation that HCV RNA detection was intermittent during early days post infection agrees with previous reports on infection experiments[7,10,33], a finding which has led the investigators to suspect the consistency of viral replication and gene expression in these cell models.

In conclusion, we report an *in vitro* system of cultured HepG2 cells infected with HCV particles. These cells support viral replication and gene expression. The consistent expression of viral proteins and the ability of culture medium to transmit the virus to new cells make this model optimum for studying HCV life cycle, screening for anti HCV drugs and testing the efficacy of therapeutic antibodies.

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**S- Editor** Wang J **L- Editor** Wang XL **E- Editor** Bi L

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*World J Gastroenterol* 2006 August 14; 12(30): 4843-4849
***World Journal of Gastroenterology*** ISSN 1007-9327


*BASIC RESEARCH*

# Expression of tissue factor in pancreatic adenocarcinoma is associated with activation of coagulation

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Supported by the Ministry of Science, State of Mecklenburg; Grant No. RVFF UR40 to J Matthias Löhr and Dr. Robert-Pfleger Stiftung to J Matthias Löhr, Bamberg, Germany
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Received: 2005-08-23 Accepted: 2005-10-10

## Abstract

**AIM:** To study expression of tissue factor (TF) in pancreatic cancer and its role in the development of thromboembolism.

**METHODS:** TF expression was studied in eight human pancreatic carcinoma cell lines by Northern blot and indirect immunofluorescence. Expression of alternatively spliced TF (asTF) was assessed by RT-PCR. In addition, TF expression was determined by immunofluorescence in pancreatic tissues of 19 patients with pancreatic adenocarcinoma (PCa), 9 patients with chronic pancreatitis (CP) and 20 normal controls. Plasma samples (30 PCa-patients, 13 CP-patients and 20 controls) were investigated for soluble TF levels and coagulation activation markers [thrombin-antithrombin Ⅲ complex (TAT), prothrombin fragment 1 + 2 (F1 + 2)].

**RESULTS:** All pancreatic carcinoma cell lines expressed TF (8/8) and most of them expressed asTF (6/8). TF expression at the protein level did not correlate with the differentiation of the carcinoma cell line. All but two pancreatic cancer tissue samples stained positive for TF (17/19). In all samples of CP weak staining was restricted to pancreatic duct cells, whereas only a few subendothelial cells were positive in 9/20 of normal controls. TF and TAT levels in PCa patients were significantly elevated compared to controls whereas elevated F1 + 2 levels did not reach statistical significance compared to controls. In CP patients TAT and F1 + 2 levels proved to be significantly elevated compared to controls, although TAT elevation was less pronounced than in PCa patients.

**CONCLUSION:** We conclude that in addition to the upregulated expression of TF on the cell membrane, soluble TF might contribute to activation of the coagulation system in pancreatic cancer.



**Key words:** Coagulation activation; Pancreatic carcinoma; Thromboembolism; Thrombosis; Tissue factor

Haas SL, Jesnowski R, Steiner M, Hummel F, Ringel J, Burstein C, Nizze H, Liebe S, Löhr JM. Expression of tissue factor in pancreatic adenocarcinoma is associated with activation of coagulation. *World J Gastroenterol* 2006; 12(30): 4843-4849

http://www.wjgnet.com/1007-9327/12/4843.asp

## INTRODUCTION

Cancer patients are highly susceptible to thromboembolic complications. These thromboembolic complications include venous and arterial thrombosis, migratory thrombophlebitis, pulmonary embolism and disseminated intravascular coagulation (DIC). Idiopathic deep vein thrombosis may be the first clinical manifestation of an occult malignancy and the cancer risk is particularly increased within the first twelve months after the diagnosis of thromboembolism[1].

Patients with idiopathic venous thrombosis have been recognized to have a three to four fold increased likelihood of harboring malignancies[2,3]. Cancer patients have a two to eight fold higher risk of dying after an acute thrombotic event than patients without cancer[4-6]. Furthermore, pancreatic cancer is associated with the highest risk for thromboembolism with an estimated risk ratio of 10[7].

Although the first description of an association of malignant disease with thrombotic events dated back as early as 1865, the underlying mechanisms are poorly understood[8].

Tissue factor (TF) is a key element in the initiation of the extrinsic coagulation cascade. Binding of activated factor Ⅶ to TF results in the activation of factor IX and X ultimately leading to thrombin formation which generates fibrin from fibrinogen and activates platelets[9]. TF is a 49-kD transmembrane glycoprotein belonging to the cytokine receptor family group 2 and contains 263 amino acid residues. Procoagulant activity of TF is tightly regulated at the transcriptional level and the natural inhibitor tissue factor pathway inhibitor (TFPI) can inhibit uncontrolled activation of coagulation. Recently, a variant of TF was identified which results from alternative splicing of the primary RNA transcript. Alternatively spliced TF (asTF) proves to be biologically active[10,11].

Constitutive TF expression is demonstrated predominantly in the brain, lung, placenta, cerebral cortex and kidney[12,13]. Aberrant expression of TF, the principal initiator of blood coagulation, has been postulated to contribute to thrombosis in cancer patients.

Various tumor entities are shown to express TF, including glioma[14], breast cancer[15,16], non-small cell lung cancer[17,18] and pancreatic cancer[19,20].

The present study was designed to systematically investigate the expression of TF by pancreatic carcinoma cells *in vitro* and *in vivo*. Specifically, we focused on the following questions: Do ductal pancreatic adenocarcinoma cells express TF and is the expression intensity related to the degree of tumor differentiation? Is the expression of TF restricted to pancreatic carcinoma cells? Does expression of TF adversely affect blood coagulation in patients as analysed by coagulation activation markers? Is clinical thromboembolism in these patients related to plasma coagulation activation?

## MATERIALS AND METHODS

### Cell lines

Eight human pancreatic carcinoma cell lines were studied for TF expression: AsPC-1, BxPC-3, Capan-1, Capan-2, PaCa-2, PaCa-3, PaCa-44, and PANC-1. The cell lines have been previously studied and characterized[21-23]. The grading of the tumor cell lines as assessed by electron microscopy[21] is listed in Table 1. The expression of asTF was assessed in eight pancreatic cancer cell lines (AsPC-1, BxPC-3, Capan-1, Capan-2, PaCa-44, PANC-1, NP9, NP29), in two colorectal carcinoma cell lines (DLD-1 and SW48), in the cervical cancer cell line HeLA and in fibroblasts.

Cells were grown in their respective medium (usually RPMI-1640) supplemented with 10% fetal calf serum. For serum deprivation, cells were seeded in tissue culture flasks and incubated with medium containing 10% fetal calf serum for 24 h, then washed twice with sterile PBS, and finally incubated with serum-free medium.

### Northern blot

RNA was extracted from exponentially growing cell lines as described earlier[22]. Twenty μg of total RNA was analysed by formamide agarose gel electrophoresis and Northern blotting[22]. Blots were hybridized to a TF cDNA probe[24] and subsequently to a ribosomal cDNA control probe (S138). The 1.4 kb TF-specific insert was labelled with 32P-dATP yielding 5-7 × $10^8$ cpm per μg DNA. Filters were hybridized with 1-2 × $10^6$ cpm/mL hybridization mix at 42℃ overnight, washed with 2 × SSC at 37℃ for 30 min and 0.5 × SSC at 55℃ for 10 min, dried, and exposed to Kodak XAR film using Kronex lightning enhancer screens. Films were evaluated semiquantitatively according to an established rating. A minimal or no signal representing densitometric measurements < 0.100 was rated 0; a weak signal equivalent to densitometric values 0.100 to 1.0 was rated 1; a signal equivalent to densitometric values 1.0 to 5.0 was rated 2, and a signal equivalent to densitometric readings > 5.0 was rated 3[21,22].

**Table 1 Tissue factor expression in eight human pancreatic adenocarcinoma cell lines in relation to the histological differentiation determined by northern-blot analysis (RNA level) and by indirect immunofluorescence (protein level)**

| | Grade | RNA | Protein |
|---|---|---|---|
| Capan-1 | Ⅰ | 1[1] | 2 |
| Capan-2 | Ⅰ-Ⅱ | 3 | 2 |
| AsPC-1 | Ⅰ-Ⅱ | 3 | 3 |
| BxPC-3 | Ⅱ | 3 | 2-3 |
| PANC-1 | Ⅱ-Ⅲ | 1 | 1-2 |
| PaCa-2 | Ⅱ-Ⅲ | 1 | 2-3 |
| PaCa-3 | Ⅲ | 1[1] | 3 |
| PaCa-4 | Ⅲ | 1 | 1-2 |

[1]Positive only in the absence of fetal calf serum.

**Table 2 Overview of oligonucleotides used for amplification of TF and asTF**

| Type of primer | Oligonucleotide |
|---|---|
| Forward | 5′-CAGGCACTACAAATACTGTGGCAG-3′ |
| Reverse | 5′-TGCAGTAGCTCCAACAGTGCTTCC-3′ |

TF: Tissue factor; asTF: Alternatively spliced TF.

### RT-PCR

For detection of concomitant expression of TF and asTF, cells were washed with PBS. After complete removal of the PBS, 350 μL buffer RLT (RNeasy Mini Kit; Qiagen, Hilden, Germany) plus β-ME (10 μL/mL) was pipetted directly onto the cells. The lysate was processed further according to the manufacturer´s recommendations. Isolated total RNA (1 μg, determined photometrically) was reverse transcribed using Superscript Ⅱ reverse transcriptase (Invitrogen; Karlsruhe, Germany) and oligo-dT primers. The resulting cDNA was amplified in separate tubes using Hot Star Taq (Qiagen; Hilden, Germany). Used primers are listed in Table 2. Oligonucleotides corresponded to TF nucleotides 221-244 and 1013-1036, respectively. TF and asTF were discriminated on a 2% agarose gel as bands of 815 and 656 bp, respectively. The cycling parameters are as follows: initial denaturation and Taq polymerase activation at 94℃ for 15 min, cycling (33 ×) at 94℃ for 45 s, 50℃ for 45 s and 72℃ for 45 s and a final extension at 72℃ for 5 min.

### *Immunofluorescence*

Tumor cell lines grown on glass slides, cryostat sections of pancreatic carcinoma, chronic pancreatitis, and of normal pancreatic tissue were fixed in acetone at -20℃ for 10 min and immunostained by indirect immunofluorescence. The primary mouse monoclonal antibody against TF was clone 5G9[25]. Staining was performed on at least two separate preparations for all tissue sections or cell lines. Staining intensity was graded semiquantitatively as described before[21,22]: a negative staining was rated 0, weakly positive 1, moderately positive 2, and strongly positive 3. This referred to the overall staining intensity. If there were focal positive spots, it was documented separately. Appropriate controls were included throughout this investigation.

### *Pancreatic tissue*

Forty-eight tissue samples were available for further investigation: 19/30 samples from patients with pancreatic carcinoma and 9/13 samples from patients with chronic pancreatitis. Twenty normal tissue samples were obtained from organ donor pancreata[26]. After surgical removal the tissue sample was immediately snap frozen in liquid nitrogen. Five micrometer thick sections were stained with hematoxylin and eosin for histological examination[27].

### *Patients*

The study population comprised 30 patients with pancreatic ductal adenocarcinoma (PCa). Thirteen patients with chronic pancreatitis (CP) and 30 healthy subjects (co) served as controls. Clinically overt thromboembolic events were noted in four patients.

With patients' written informed consent citrate-anticoagulated blood was drawn on the first day of hospitalization prior to the administration of medications potentially interfering with the coagulation system. Platelet-free plasma aliquots were stored at -80℃.

All patients underwent extensive diagnostic work-up including abdominal ultrasound, contrast-enhanced computed tomography and magnetic-resonance tomography, respectively. Suspected thromboembolic manifestations were investigated by doppler ultrasound and when necessary with computed tomography and angiography.

### *Coagulation studies*

Separate plasma aliquots obtained from patients were quickly thawed and utilized for the following commercially available assays according to the instructions provided by the manufacturers. TF antigen concentration was measured using the Imubind® Tissue Factor ELISA kit (American Diagnostics, Greenwich, CT, USA). Thrombin-antithrombin III complex (TAT) was quantitated employing the Enzygnost® TATmicro enzyme immunoassay (Behring, Frankfurt, Germany). Prothrombin fragment 1 + 2 (F1 + 2) was evaluated using the Enzygnost® F1 + 2 kit (Behring, Frankfurt, Germany).

### *Statistical analysis*

Plasma levels of TF, F1 + 2 and TAT are expressed as the means ± SD. Statistical significance was calculated using the two-sided Mann-Whitney test. A $P$-value of $< 0.05$ was considered statistically significant.

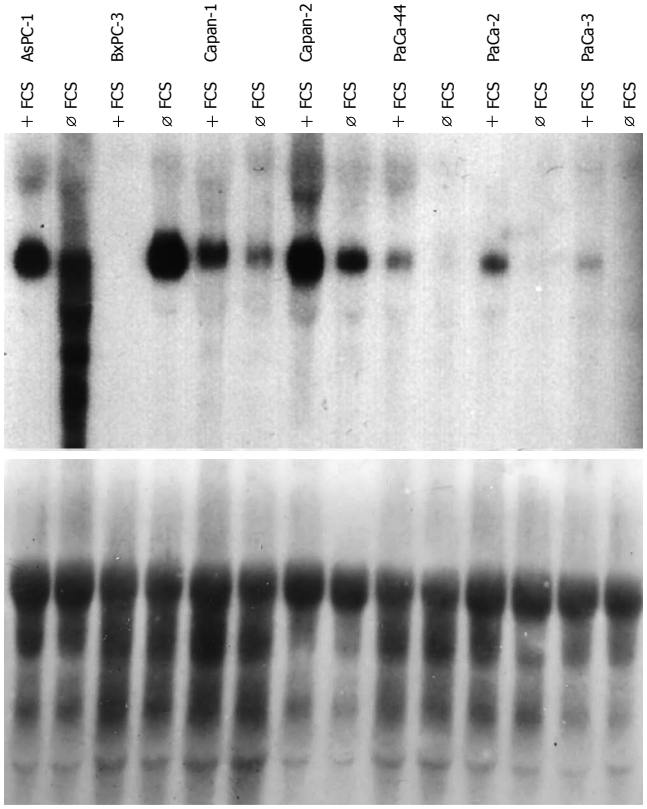


**Figure 1** Northern blot of human pancreatic carcinoma cell lines utilizing a cDNA probe for tissue factor (TF) and a ribosomal cDNA control probe (S138). Cell culture conditions with and without (ø) fetal calf serum (FCS).

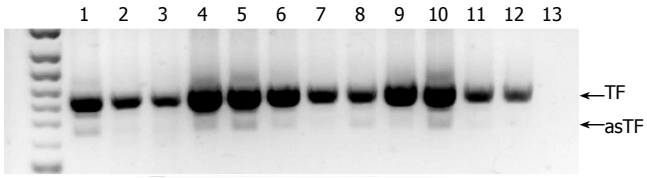


**Figure 2** Assessment of TF and asTF expression by RT-PCR. 1: DLD-1; 2: SW48; 3: PANC-1; 4: BxPC-3; 5: PaCa-44; 6: Capan-2; 7: Capan-1; 8: AsPC-1; 9: NP9; 10: NP29; 11: HeLa; 12: Fibroblasts; 13: Negative control.

## RESULTS

### *Expression of TF in pancreatic carcinoma cell lines*

All of the eight human pancreatic carcinoma cell lines expressed TF (Table 1, Figures 1, 2, 3A and B). Expression at RNA-level could be modulated in part by serum depletion (Figure 1). All lowly differentiated cell lines (grade Ⅱ and Ⅱ-Ⅲ) exhibited weak TF expression determined by Northern-blot analysis, whereas three of the four highly differentiated cell lines (grade Ⅰ, Ⅰ-Ⅱ and Ⅱ) were found to have a strong TF expression at the RNA level. In contrast, no clear correlation between differentiation and TF protein levels could be demonstrated by immunofluorescence (Table 1).

### *Expression of asTF in pancreatic cancer cell lines*

In addition to TF, the majority of studied pancreatic carcinoma cell lines (6/8) expressed asTF (Figure 2). Furthermore, asTF expression could be demonstrated in the colorectal carcinoma cell lines DLD-1. The colorectal

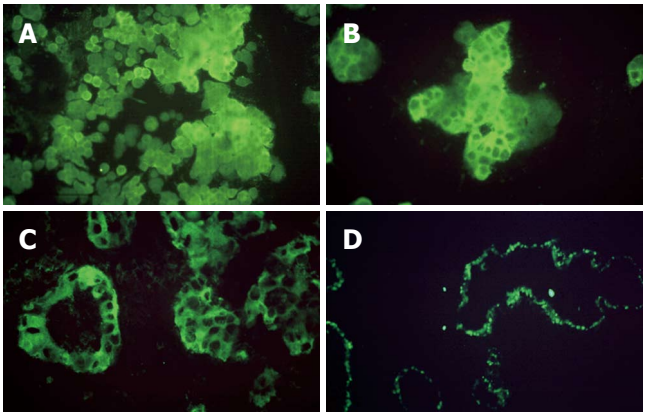


**Figure 3** Representative demonstration of tissue factor (TF) expression by immunofluorescence in AsPC-1 (**A**) and CAPAN-1 (**B**) pancreatic cancer cell lines, in tissue of pancreatic cancer (**C**) and chronic pancreatitis (**D**).

**Table 3 Immunofluorescence staining analysis of tissue factor expression in patients with pancreatic carcinoma (PCa), chronic pancreatitis (CP) and in healthy controls (Co)**

| | PCa ($n$ = 19) | CP ($n$ = 9) | Co ($n$ = 20) |
|---|---|---|---|
| Total immunoreactivity | 17 (84%)[1] | 9/9 (100%)[2] | 9/20 (45%)[3] |
| Grade 3 | 4 | 0 | 0 |
| Grade 2 | 7 | 0 | 0 |
| Grade 1 | 6 | 9[+] | 0 |
| Negative | 2 | 0 | 11 |

[1]$P$ = 0.027 *vs* controls; [2] A few positively stained epithelial duct cells; [3] Expression restricted to subendothelial cells.

carcinoma cell line SW48, the cervical cancer cell line HeLA and fibroblasts did not express asTF.

### *Expression of TF in pancreatic tissue*

Of the 19 pancreatic carcinoma tissue samples nearly all (17/19) were positive for TF (4/19: grade 3, 7/19: grade 2, 6/19: grade 1) and only two were negative (Table 3, Figure 3C). The amount of TF expression in tumor tissue did not correlate with the grading of the tumor (Table 3).

All tissue samples obtained from chronic pancreatitis demonstrated positive staining of epithelial pancreatic duct cells (Table 3, Figure 3D). However, staining intensity was rated weakly in these cases. Nearly half of all normal control tissue samples exhibited only weak staining of a few subendothelial cells (9/20) (Table 3).

### *Plasma concentrations of TF in patients with pancreatic cancer and healthy controls*

TF plasma concentrations in 30 patients with pancreatic cancer were significantly enhanced versus 30 healthy controls (288 ± 41.4 ng/L *vs* 139 ± 9.7 ng/L, $P$ = 0.005) (Figure 4).

### *Coagulation studies*

Plasma levels of TAT in patients with pancreatic carcinoma (PCa) were more than twenty-times higher than in normal controls (89.4 ± 38.8 μg/L *vs* 4.0 ± 3.5 μg/L, $P$ = 0.002). Interestingly, they were also significantly enhanced

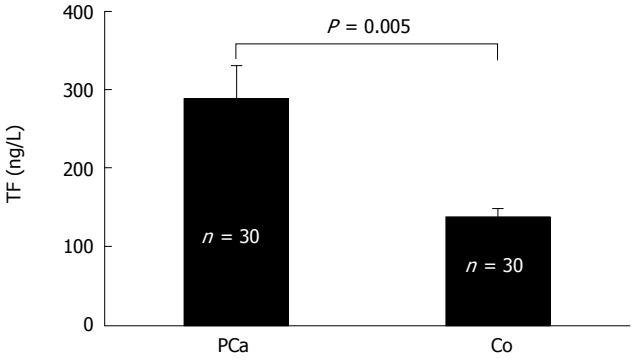


**Figure 4** Plasma concentrations of tissue factor (TF) in patients with pancreatic cancer (PCa) versus controls (Co). Bars indicate means and standard deviation.

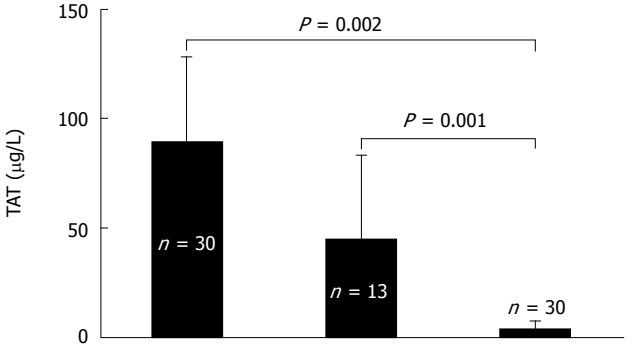


**Figure 5** Plasma concentrations of thrombin-antithrombin complex (TAT) in patients with pancreatic cancer (PCa), chronic pancreatitis (CP) and healthy controls (Co). Bars indicate means and standard deviation.

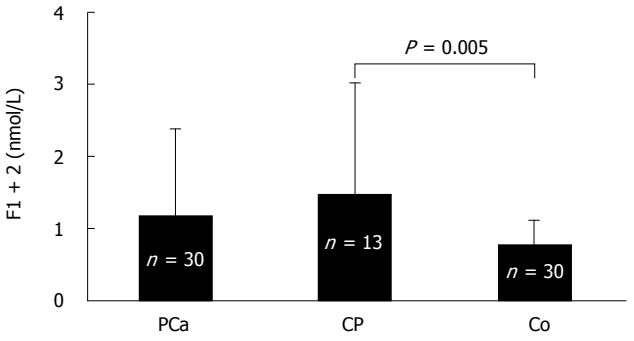


**Figure 6** Plasma concentrations of prothrombin fragment 1 + 2 (F1 + 2) in patients with pancreatic cancer (PCa), chronic pancreatitis (CP) and healthy controls (Co). Bars indicate means and standard deviation.

in patients with chronic pancreatitis (CP) versus healthy controls (45.1 ± 37.9 μg/L *vs* 4.0 ± 3.5 μg/L, $P$ = 0.001) (Figure 5). However, higher levels in PCa patients compared to CP patients did not reach statistical significance.

Plasma levels of prothrombin F1 + 2 proved to be higher in patients with pancreatic adenocarcinoma although not reaching statistical significance (1.16 ± 1.56 nmol/L *vs* 0.75 ± 0.34 nmol/L, $P$ = 0.16), whereas significant elevation of F1 + 2 was observed in patients with chronic pancreatitis versus controls (1.46 ± 1.23 nmol/L *vs* 0.75 ± 0.34 nmol/L, $P$ = 0.005) (Figure 6). There was no correlation between the plasma levels of

**Table 4 Patients with pancreatic carcinoma complicated by clinically overt thromboembolism**

| Case | Sex/age | Stage[1] | Grade | TF[2] | TF (pg/mL) | Location of thromboembolism |
|---|---|---|---|---|---|---|
| 1 | m/65 | 4 | II | 3 | 291 | Splenic vein thrombosis |
| 2 | m/69 | 4 | II | 2 | nd | Mesenteric artery thrombosis |
| 3 | f/61 | 4 | II | 1[3] | 391 | Upper jugular vein thrombosis |
| 4 | m/62 | 4 | II | 2 | nd | Pulmonary embolism |

[1] Staging according to UICC; [2] Results from immunohistochemistry, i.e. immunofluorescence for TF; [3] TF was focally positive; nd: not determined.

TAT or F1 + 2 and the extent of TF expression in the pancreatic tumor tissue (data not shown). However, there was a strong correlation between TAT and F1 + 2 plasma levels ($P < 0.05$).

### *Patients with pancreatic cancer and thromboembolic complications*

Four of the TF-positive patients had thrombosis, as revealed by doppler ultrasound, computed tomography and/or angiography (Table 4). All patients with thrombosis had a stage 4 pancreatic carcinoma. In two cases TF plasma levels were available which were increased by a factor of 2 and 2.8, respectively, compared to the mean value of TF plasma levels in healthy controls.

## DISCUSSION

Our study proved expression of TF by epithelial tumor cells in pancreatic adenocarcinoma *in vitro* and *in vivo*. In patients with pancreatic cancer, a hypercoagulative state could be confirmed by increased concentrations of the TAT in conjunction with elevated TF plasma levels. Four patients expressing TF in their tumors developed clinically overt thromboembolism.

The presence of a malignant disease dramatically increases the risk for thromboembolic events. Immobility, indwelling catheters, surgical procedures and chemotherapy represent risk factors rendering cancer patients for thromboembolism[28]. In addition, direct activation of the coagulation cascade by cancer cells is considered a key feature of the well-established increased risk for thrombotic events. TF plays a central role in activating coagulation and enhanced expression is implicated in diseases with enhanced thrombotic features like cancer[29], atherosclerosis[30], sickle cell disease[31] and sepsis[32].

We analyzed the expression of TF in eight well characterized cell lines of ductular adenocarcinoma[23]. Without exception all showed TF expression at RNA and protein levels. Highest RNA levels were found in the well-differentiated cancer cell lines (Capan-2, AsPC-1, BxPC-3). However, in relation to differentiation no clear correlation of TF expression could be observed at the protein level. The fact that the amount of TF protein levels did not strictly correspond to RNA levels suggests that posttranscriptional regulation might be of importance in TF expression. We could not confirm the finding of Kakkar that expression of TF was most prominent in poorly differentiated tumors[20].

In addition to expression of TF, the majority of studied pancreatic carcinoma cell lines expressed the recently characterized asTF[10,11]. This splice variant contains the extracellular domain of TF, but lacks the transmembrane and cytoplasmatic domain. Exon 4 is spliced directly to exon 6 leading to a frameshift so that the translated peptide comprises residues 1-166 of the extracellular domain of TF and a unique C terminus (residues 167-206). AsTF proved to have full pro-coagulant activity. The expression of asTF might contribute to systemic hypercoagulopathy in pancreatic cancer.

Corresponding to the TF expression in pancreatic carcinoma cell lines we demonstrated, in the majority of tissue specimens of patients with pancreatic cancer, TF expression in tumor cells, which showed a high variability in the expression rate determined by immunofluorescence. Recently, upregulated TF expression in colorectal cancer cells was ascribed to *K-ras* mutations[33]. As mutations in the *K-ras* oncogene are an early event in the development of pancreatic cancer and as these mutations are found in the majority of pancreatic cancer patients, mutational activation of *K-ras* might have a contributory role in upregulated TF expression[34]. Further studies are warranted to clarify whether inactivation of the tumor suppressor gene PTEN and hypoxia are involved in enhanced TF expression in pancreatic cancer as it was recently demonstrated in glioblastoma[35]. Lately, Nitori and co-workers determined TF expression in pancreatic cancer by immunohistochemistry and found an association of high TF expression with metastasis and a low survival rate[36].

To our knowledge this is the first report demonstrating an enhanced TF expression in tissues of patients with chronic pancreatitis which was confined to pancreatic duct cells. This was a surprising result as tissue of chronic pancreatitis patients was initially intended to be included in the study to serve as a negative control. Previous data have shown that the exposure of endotoxin, tumor necrosis factor (TNF)-$\alpha$ and interleukin (IL)-1 results in upregulation of TF expression *in vitro*[37-40]. Proinflammatory cytokines secreted by the inflammatory infiltrate in chronic pancreatitis might be an explanation for the upregulated TF expression[41]. In contrast, only weak staining of a few subendothelial cells was found in control tissue specimens. Upregulated TF expression in the tissue of chronic pancreatitis patients might have a contributory role in the development of thrombotic events, which is a well-known complication in chronic pancreatitis.

TF plasma concentrations in patients with pancreatic cancer were found to be more than twice as high than in normal controls which might stem from asTF or from TF-containing microparticles and from TF which is secreted by activated leukocytes, respectively[42-44]. Further studies are needed to prove that the source of TF in the plasma of patients with pancreatic cancer is the tumor itself.

In parallel to enhanced TF expression, markers of thrombin formation (TAT, F1 + 2) were elevated in the plasma of patients with pancreatic cancer and chronic pancreatitis. A procoagulatory state in patients with chronic pancreatitis might facilitate the generation of portal and splenic vein thrombosis, which is commonly seen in this patients' group.

Four patients with pancreatic cancer had concurrent venous thrombosis or pulmonary embolism. TF expression was highly variable but all patients had a stage 4 carcinoma. Apparently the tumor load and tumor progression is a more powerful prognostic marker for thrombotic complications than TF expression in pancreatic cancer. This is in accordance with data showing that the stage of colorectal cancer most consistently correlates with the risk for thromboembolism[45].

In this study we focused on TF expression, taking into account that undoubtedly multiple factors modulate hypercoagulative state.

Tissue factor pathway inhibitor (TFPI) is an important factor limiting the effects of TF. In a recent study low levels of TFPI were associated with an increased risk of venous thrombosis[46]. Mice deficient of TFPI exhibited an increased rate of thrombosis and accelerated atherosclerosis[47]. Both pancreatic carcinoma and chronic pancreatitis produce a rich stroma[48]. We demonstrated that epithelial tumor cells can synthesize and deposit extracellular matrix proteins in pancreatic carcinoma[21,49]. Fibronectin itself which is expressed by pancreatic tumor cells[21] is capable of activating the coagulation cascade[50].

In addition to its haemostatic potential, the TF expression by tumor cells has implications in tumor biology[51,52]. Belting and coworkers have shown that TF directly promotes angiogenesis through protease-activated receptor-2 (PAR-2) signaling[53]. Upregulation of the urokinase receptor in pancreatic cancer by TF enhances tumor invasion and metastasis[54]. Pancreatic carcinoma cells possess a functioning thrombin receptor, and stimulation with thrombin representing the product of the coagulation cascade causes cell proliferation[55]. Furthermore, thrombin has a proangiogenic function[56,57].

In summary, enhanced TF expression in pancreatic adenocarcinoma in conjunction with elevated TF plasma levels might have a crucial role in hypercoagulopathy, resulting in thromboembolism.

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**S- Editor** Wang J **L- Editor** Zhu LH **E- Editor** Ma WH

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*World J Gastroenterol* 2006 August 14; 12(30): 4850-4858
***World Journal of Gastroenterology*** ISSN 1007-9327


*BASIC RESEARCH*

# DA-9601, a standardized extract of *Artemisia asiatica*, blocks TNF-α-induced IL-8 and CCL20 production by inhibiting p38 kinase and NF-κB pathways in human gastric epithelial cells

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Supported by grants from the Korea Health 21 R&D Project, Ministry of Health and Welfare, No.01-PJ3-PG6-01GN09-003, and the Korea Food and Drug Administration, No. 05142-620
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Received: 2006-04-10 Accepted: 2006-05-25

## Abstract

**AIM:** To investigate whether, or how, DA-9601, which is a new gastroprotective agent, inhibits TNF-α-induced inflammatory signals in gastric epithelial AGS cells.

**METHODS:** Cell viability was determined by MTT assay. IL-8 and CCL20 promoter activities were determined by a luciferease reporter gene assay. NF-κB-dependent transcriptional activity was determined by I-κBα degradation, NF-κB p65 nuclear translocation and a luciferase activity assay. IL-8 and CCL20 gene expression and protein secretion were determined by RT-PCR and an enzyme-linked immunosorbent assay (ELISA). Total and phosphorylated forms of mitogen-activated protein kinases (MAPKs) were determined by Western blot.

**RESULTS:** Treatment of AGS cells with DA-9601 reduced TNF-α-induced IL-8 and CCL20 promoter activities, as well as their gene expression and protein release. TNF-α also induced NF-κB-dependent transcriptional activity in AGS cells. In contrast, in cells treated with DA-9601, TNF-α-induced NF-κB activity was significantly blocked. Although all three MAP kinase family members were phosphorylated in response to TNF-α, a selective inhibitor of p38 kinase SB203580 only could inhibit both NF-κB-dependent transcriptional activity and IL-8 and CCL20 production, suggesting a potential link between p38 kinase and NF-κB-dependent pathways in AGS cells. Interestingly, DA-9601 also selectively inhibited p38 kinase phosphorylation induced by TNF-α.

**CONCLUSION:** DA-9601 blocked TNF-α-mediated inflammatory signals by potentially modulating the p38 kinase pathway and/or a signal leading to NF-κB-dependent pathways in gastric epithelial cells.



**Key words:** CCL20; IL-8; *Artemisia asiatica*; DA-9601; TNF-α; p38 kinase; NF-κB

Choi SC, Choi EJ, Oh HM, Lee SG, Lee JK, Lee MS, Shin YI, Choi SJ, Chae JR, Lee KM, Lee WJ, Park JS, Shin CY, Oh TY, Jun CD. DA-9601, a standardized extract of *Artemisia asiatica*, blocks TNF-α-induced IL-8 and CCL20 production by inhibiting p38 kinase and NF-κB pathways in human gastric epithelial cells. *World J Gastroenterol* 2006; 12(30): 4850-4858

http://www.wjgnet.com/1007-9327/12/4850.asp

## INTRODUCTION

*Artemisia asiatica* has been frequently used in traditional Asian medicine for the treatment of diseases such as inflammation, cancer and microbial infection. Along this line, a novel antipeptic formulation prepared from the ethanol extracts of *A. asiatica*, namely DA-9601 (Stillen™), has been reported to posses anti-oxidative and anti-inflammatory activities in experimentally induced

gastrointestinal damage as well as hepatic and pancreatic lesions[1-4]. DA-9601 is now on the market in South Korea and will be on sale in other Asian countries in the near future. However, despite studies in animals and humans, the detailed cellular mechanism of the pharmacologic actions of DA-9601 is largely unknown.

Chemokines are potential mediators that in many cases can act as a signal for the emigration of blood cells. The C-X-C chemokines are considered the most important mediators for the accumulation of granulocytes[5,6]. One member of this cytokine family, chemokine interleukin-8 (IL-8), has been shown to be elevated in gastric biopsy samples of patients with *H pylori*-associated gastritis[5], and is considered to be an important mediator for the initiation of host innate immunity by recruiting granulocytes[7]. On the other hand, CCL20 is a recently described C-C chemokine (also known as a liver- and activation-regulated chemokine or macrophage inflammatory protein 3α) that was first identified by screening the GenBank database of expressed sequence tags for novel chemokine molecules[8]. CCL20 is also expressed in gastric epithelial cells, upregulated by infection with *H pylori*, and implicated in the initiation of host adaptive immunity by regulating recruitment of dendritic cells[9], effector memory T cells and B cells via CCR6[10]. Given their potential importance in inflammatory responses, these two chemokines may be good target systems in evaluating the anti-inflammatic efficacy of potential pharmacologic drugs in gastric epithelial cells.

In the current study, we primarily investigated whether TNF-α induces IL-8 and CCL20 genes, as well as their protein products, in human gastric epithelial AGS cells. Although TNF-α is a candidate factor for involvement in inflammation-mediated gastric mucosal injury, the mechanisms of action for this cytokine on gastric epithelial cells are still poorly understood. We next analyzed where DA-9601 acted in the TNF-α-induced inflammatory cascade.

## MATERIALS AND METHODS

### Reagents and Antibodies

DA-9601 (Lot No. DA-9601-L-07) with 0.42% of active ingredient, eupatilin[11], was extracted from *A. asiatica* and supplied to this study after HPLC analysis in Dong-A Pharmaceutical Co. Ltd., (Yongin, South Korea)[2]. Alkaline phosphatase-conjugated rabbit anti-goat IgG, and p-nitrophenyl phosphate tablets, dimethyl sulfoxide, phosphate-buffered saline (PBS), 3-(4, 5,-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Chemical Co. (St. Louis, MO). Recombinant human TNF-α goat anti-human IL-8 polyclonal antibody, mouse anti-human CCL20 monoclonal antibody (clone 67310.111), and goat anti-human CCL20 polyclonal antibody were obtained from R&D Systems Inc. (Minneapolis, MN). Rabbit anti-human IL-8 polyclonal antibody was from Endogen Inc. (Woburn, MA). Antibodies against p38 kinase, c-Jun N-terminal kinase (JNK), extracellular signal-regulated kinase (ERK), and the antibodies specific to the phosphorylated forms (pp38, Thr180/Tyr182; pJNK, Thr183 Tyr185; pERK1/2, Thr202/Tyr204) were purchased from Cell Signaling Technology, Inc.(Beverly, MA). SB203580, SP600125, PD98059 and PDTC were purchased from Calbiochem (La Jolla, CA). Anti-human I-κBα was from Santa Cruz Biotechnology (Santa Cruz, CA).

### Report gene construction

IL-8 promoter-luciferase reporter vector (pGL3-pIL-8) was obtained from Dr. J.-S. Chun in Gwangju Institute of Science and Technology (Korea). The CCL20 promoter from -1905 to +30 was amplified from 100 ng of human genomic DNA by PCR under standard conditions with the following primers (restriction sites underlined) pCCL20_forward(*SacI*) 5'-ATACCGAGCTCGGCCAG TCTGGTCTCGAACT-3'; pCCL20_reverse (*Hind*Ⅲ) 5'-ATACCAAGCTTCTTTAATCAATATTGCAGTT-3' and cloned into the pGL3-basic plasmid (Promega, Mannheim, Germany) to generate pGL3-pCCL20 luciferase vector. pGL3-pCCL20 was sequence verified with an ABI3700 sequencer (ABI, Foster City, CA) before use.

### Cell culture

Human gastric epithelial AGS cells and human kidney epithelial 293T were obtained from the American Type Culture Collection (ATCC). The cells were cultured at 37℃ in a 5% $CO_2$ atmosphere in RPMI-1640 supplemented with heat-inactivated 10% fetal bovine serum (FBS; GibcoBRL) and appropriate antibiotics.

### Cell viability assay

Cellular viability was evaluated by the reduction of MTT to formazan. A stock solution of MTT was prepared in phosphate-buffered saline (PBS), diluted in RPMI 1640 medium, and added to cell-containing wells at a concentration of 0.5 mg/mL after the culture medium was first removed. The plates were then incubated for 4 h at 37℃ in 5% $CO_2$. At the end of the incubation period, the medium was aspirated, and the formazan product was solubilized with dimethyl sulfoxide. Absorbency was measured on a multiscan reader with a 570 nm wavelength filter.

### IL-8 and CCL20 Measurement

The concentration of IL-8 or CCL20 in culture supernatants from AGS cells was measured by a previously described method[12,13]. In brief, 96-well microtiter plates (MaxiSorpTM, Nunc, Denmark) were coated with 2 μg/mL of goat anti-human IL-8 (R&D Systems) or mouse anti-human CCL20 (clone 67310.111; R&D Systems) in 50 μL PBS at 4℃ overnight. All further steps were carried out at room temperature. After washing three times with PBS, non-specific binding sites were blocked by incubation with 150 μL PBS, 1% BSA and 0.05% Tween 20/well for 2 h. After three washes with PBS, 50 μL of samples or standards were added and incubated for 2 h. As a second antibody, 0.5 μg/mL polyclonal rabbit anti-human IL-8 (Endogen) or polyclonal goat anti-human CCL20 (R&D Systems) was added and incubated for 2 h. As a third antibody, alkaline phosphatase-conjugated monoclonal mouse anti-rabbit IgG (for IL-8) or rabbit anti-goat IgG

(for CCL20) was diluted in 50 μL PBS 0.1% BSA and 0.05% Tween 20 to 1:50000 and incubated for 2 h. Finally, alkaline phosphatase substrate *p*-nitrophenyl phosphate (Sigma) was added at a concentration of 1mg/mL in 0.1 mol/L glycine buffer, pH 10.4, containing 1 mol/L $MgCl_2$ and 1 mol/L $ZnCl_2$. After overnight incubation, plates were read at 405 nm on a microplate reader (Molecular Devices Corp., Sunnyvale, CA). The detection limit of the ELISA was 30 pg/mL.

### RNA Isolation and RT-PCR

AGS cells ($5 \times 10^6$) were grown in 60-mm culture dish and were incubated for 16 h in a fresh medium containing stimuli as indicated. After discarding the growth medium, total RNA was isolated from cells using easy Blue (iNtRON Biotechnology, Daejon, Korea), following the manufacturer's instructions. Reverse transcription of the RNA was performed using AccuPower RT PreMix (Bioneer, Daejon, Korea). One microgram of RNA and 20 pmol primers were preincubated at 70℃ for 5 min and transferred to a mixture tube. The reaction volume was 20 μL. cDNA synthesis was performed at 42℃ for 60 min, followed by RT inactivation at 94℃ for 5 min. Thereafter, the RT-generated DNA (2-5 μL) was amplified using AccuPower® RT PreMix (Bioneer, Korea). The primers used for cDNA amplification were: 5'-ATGACTTCCAA GCTGGCCGTGGCT-3' (sense) and 5'-TCTCAGCCCT CTTCAAAAACTTCTC-3' (antisense) for IL-8[14]; 5'-ATG TGCTGTACCAAGAGTTTG-3' (sense) and 5'-TTACAT GTTCTTGACTTTTTTACTGAGGAG-3' (antisense) for CCL20[15]; 5'-CGGAGTCAACGGATTTGGTCGTAT-3' (sense), 5'-AGCTTCTCCATGGTGGTGAAGAC-3' (antisense) for GAPDH[12]. Amplification conditions were denaturation at 94℃ for 30 s, annealing at 56℃ for 30 s, and extension at 72℃ for 30 s for 30 cycles. The expected PCR products were 289 bp (for IL-8), 291 bp (for CCL20), and 306 bp (for GAPDH). PCR products were subjected to electrophoresis on 1.2 % agarose gel and were stained with ethidium bromide.

### Assessment of NF-κB-p65-EGFP nuclear translocation

AGS cells were seeded at $5 \times 10^5$ in a 4-well plate 1 d before transfection (50% cell confluency). Cells were transfected with serum- and antibiotics-free RPMI 1640 medium containing 4 μg/mL Lipofectamine 2000 reagent (Invitrogen) and 1.6 μg/mL of NF-κB p65-EGFP vector (provided by Prof. Rainder de Martin, Department Vascular Pharmacology and Thrombosis Research, University of Vienna, Austria). After 5 h of incubation, medium was replaced with RPMI 1640 medium containing 10% FBS and antibiotics. Cells were allowed to recover at 37°C for 20 h and subsequently were stimulated as indicated in the text or figures. Fluorescence images were observed under the Olympus microscopy (Melville, NY).

### Cell extract preparation and Western blot analysis

For the analysis of phosphorylated or total protein levels of mitogen-activated protein kinases (MAPKs) and the I-κB degradation, stimulated cells were rinsed twice with ice-cold phosphate-buffered saline and then lysed in ice-cold lysis buffer (50 mmol/L Tris-HCl, pH 7.4, containing 150 mmol/L NaCl, 1% Nonidet P-40, 0.1% SDS, 0.1% deoxycholate, 5 mmol/L sodium fluoride, 1 mmol/L sodium orthovanadate, 1 mmol/L 4 nitrophe-nylphosphate, 10 g/mL of leupeptin, 10 μg/mL of pepstatin A, and 1 mmol/L 4-(2 aminoethyl) Benzenee Sufonyl fluoride). Cell lysates were centrifuged at 15000 rpm for 20 min at 4℃, and the supernatant was mixed with a one-fourth volume of 4 × SDS sample buffer, boiled for 5 min, and then separated through a 12% SDS-PAGE gel. After electrophoresis, proteins were transferred to a nylon membrane by means of Trans-Blot SD semi-dry transfer cell (Bio-Rad, Hercules, CA). The membrane was blocked in 5% skim milk (1 h), rinsed, and incubated with primary antibody (for phosphorylated MAP kinases or I-κB) in TBS containing 0.05% Tween 20 (TBS-T) and 3% skim milk overnight at 4℃. Excess primary antibody was then removed by washing the membrane four times in TBS-T, and the membrane was incubated with 0.1 μg/mL peroxidase-labeled secondary antibody (against rabbit) for 1 h. Following three washes in TBS-T, bands were visualized by ECL™ Western Blotting Detection reagents and exposed to X-ray film.

### Transient transfection and luciferase activity assay

For transient transfections, AGS cell and 293T Cells were seeded at $5 \times 10^5$ in a 12-well plate 1 d before transfection (90%-95% cell confluency). Cells were transfected with serum- and antibiotics-free RPMI 1640 medium containing 4 μg/mL Lipofectamine 2000 reagent (Invitrogen) and 1.6 μg/mL of NF-κB, IL-8 (provided by Professor J.-S. Chun, Gwangju Institute of Science and Technology, Gwangju, Korea), or CCL20 luciferase reporter constructs. After 5 h of incubation, medium was replaced with RPMI 1640 medium containing 10% FBS and antibiotics. Cells were allowed to recover at 37℃ for 20 h and subsequently were stimulated as indicated in the text or figures. Cell lysates were prepared and assayed for luciferase activity using Luciferase Assay System (Promega, Madison, WI), according to the manufacturer's instructions.

## RESULTS

### TNF-α induces IL-8 and CCL20 gene expression and protein release in AGS cells

One of the key molecules mediating the gastric mucosal inflammation is the cytokine TNF-α[16]. We therefore first examined whether TNF-α induces inflammatory signals in AGS cells. We were primarily interested in two chemokines; i.e., IL-8 and CCL20, because these proteins play central roles in the evocation of host innate and adaptive immunity[10,17]. Treatment of AGS cells with TNF-α markedly induced IL-8 and CCL20 secretion (Figure 1). The effect of TNF-α was concentration-dependent in the range of 0-10 ng/mL, as assessed by ELISA and RT-PCR. We chose 5 ng/mL of TNF-α for the following experiments as this concentration is enough for maximal induction of IL-8 and CCL20 (Figure 1A and C). Time-dependent experiments revealed that treatment with TNF-α led to rapid induction of IL-8 and CCL20 mRNAs (about 4 h after stimulation) while their

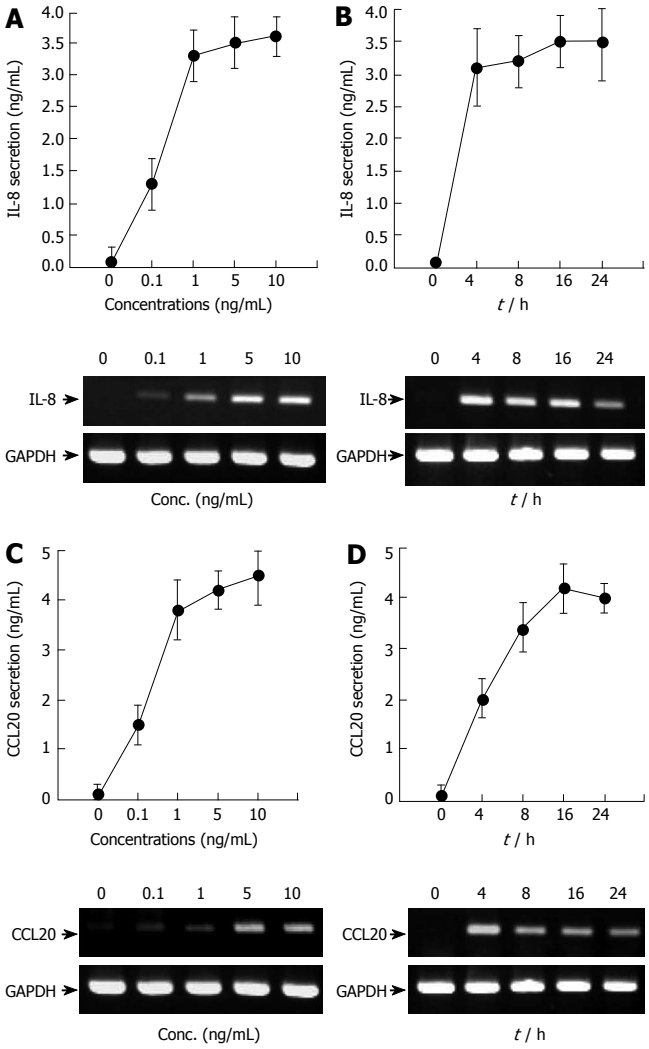


**Figure 1** TNF-α induces IL-8 and CCL20 secretion and mRNA accumulation in AGS cells in a time- and dose-dependent manner. (**A** and **C**) AGS cells (5 x $10^5$/well) were treated for 16 h with the indicated concentrations of TNF-α (0-10 ng/mL). After incubation, the supernatants were collected, and the levels of IL-8 (**A**) and CCL20 (**C**) were determined by ELISA (top). At the same time, the cells were colleted and the expression of two chemokines was determined by RT-PCR (bottom). (**B** and **D**) AGS cells (5 x $10^5$/well) were treated with TNF-α (5 ng/mL) for the indicated time points (0-24 h). IL-8 and CCL20 secretion and mRNA accumulation were determined as described above. For ELISA, results are expressed as means ± SD of three independent experiments.

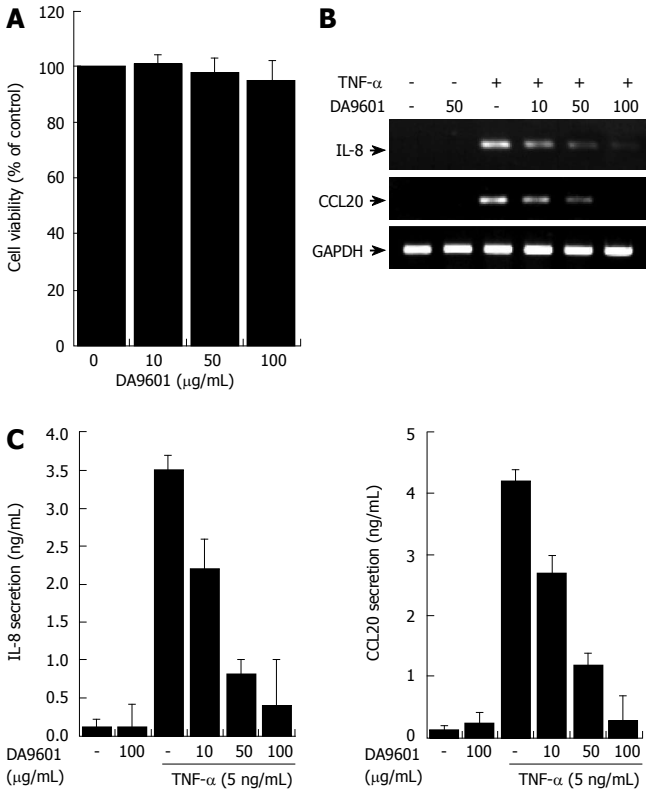


**Figure 2** DA-9601 inhibits the expression and secretion of CCL20 in AGS cells. (**A**) AGS cells (1 x $10^5$) were treated with various concentrations of DA-9601 (0-100 μg/mL) for 16 h. Quantitative analysis of cell viability was determined by the MTT assay (mean ± SD., $n$ = 3). (**B**) Cells (5 x $10^5$) were pretreated with various concentrations of DA-9601 (0-100 μg/mL) for 1 h, and then the cells were further incubated for 8 h with TNF-α (5 ng/mL). Levels of IL-8 and CCL20 mRNAs were determined by RT-PCR. (**C**) Cells (5 x $10^5$) were pretreated with various concentrations of DA-9601 (0-100 μg/mL) for 1 h, and then the cells were further incubated for 16 h with TNF-α (5 ng/mL). IL-8 and CCL20 protein levels were determined by ELISA. These data are representative of three independent experiments.

protein secretions were slightly delayed (about 16 h after stimulation) in AGS cells. Overall, we conclude that AGS cells produce IL-8 and CCL20 in response to TNF-α.

### *DA-9601 inhibits TNF-α-induced IL-8 and CCL20 gene expression and protein release in AGS cells*

According to previous reports the main effect of DA-9601 is associated with cell death or apoptosis in the rat model of cerulein-induced pancreatitis[1]. To test preliminarily whether DA-9601 affects viability of human gastric epithelial AGS cells, we performed a MTT assay. The concentrations that ranged from 0 to 100 μg/mL of DA-9601 showed no toxic effects on AGS cells at 16 h of incubation, while higher concentrations of DA-9601 (>100 μg/mL) induced delayed cytotoxicity after 24 h (Figure 2A and data not shown). There were no detectable apoptotic nuclei in DA-9601 (0-100 μg/mL)-treated cells, as verified by DAPI staining (data not shown).

RT-PCR revealed that DA-9601 (0-100 μg/mL), which alone did not induce any significant changes, significantly attenuated TNF-α (5 ng/mL)-dependent expression of IL-8 and CCL20 mRNA in human AGS cells (Figure 2B). Addition of DA-9601 dramatically reduced TNF-α-induced IL-8 and CCL20 secretions as well in a dose-dependent manner (Figure 1C). The concentration of 100 μg/mL of DA-9601 maximally inhibited the secretion of both chemokines; i.e., IL-8 and CCL20 (Figure 1C). However, as this concentration revealed weak cytotoxicity after 24 h of treatment (data not shown), we therefore chose 50 μg/mL of DA-9601 for the following experiments, unless otherwise indicated.

### *DA-9601 inhibits TNF-α-induced IL-8 and CCL20 promoter activities in both HEK293T cells and AGS cells*

To investigate whether the inhibition of both chemokine secretions by DA-9601 is due to the direct down-regulation of promoter activity, we performed the luciferase reporter gene assay for IL-8 and CCL20 promoters. As shown in Figure 3, treatment with TNF-α significantly induced IL-8 and CCL20 promoter activities (promoter-dependent luciferase signals) in both HEK293T cells and AGS cells.

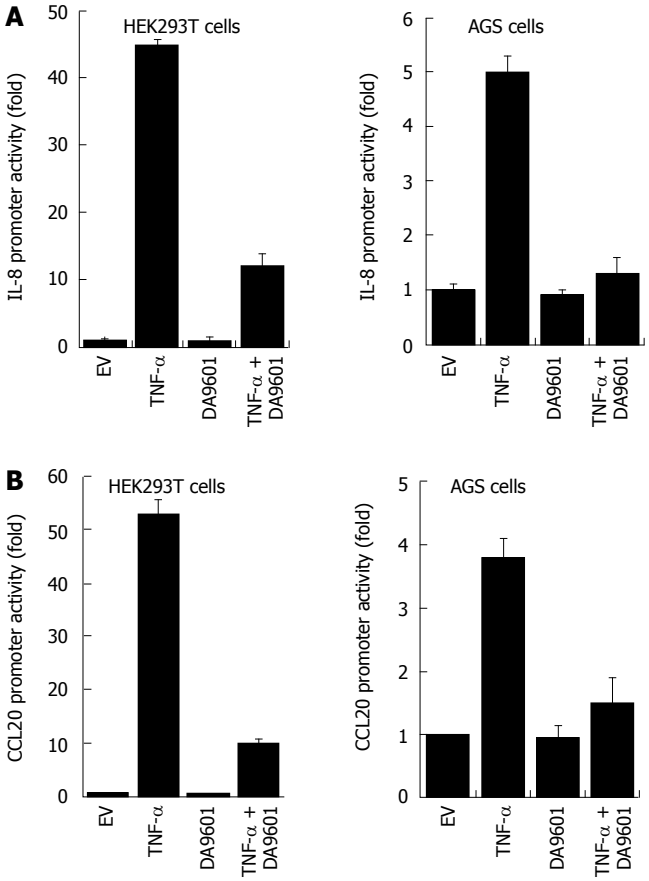


**Figure 3** DA-9601 blocks IL-8 and CCL20 promoter activities in HEK293T and AGS cells. HEK293T (left) and AGS (right) cells were transfected with pGL3-pIL-8 (**A**) or pGL3-pCCL20 (**B**) luciferase vectors. After 24 h of incubation, cells (1 x $10^5$) were pre-treated for 1 h with DA-9601 (50 μg/mL) and stimulated for additional 16 h with medium alone or medium containing TNF-α (5 ng/mL). At the end of incubation, cells were lysed, and the relative luciferase activity was measured using Luciferase Assay System. Results are expressed as means ± SD of three independent experiments.

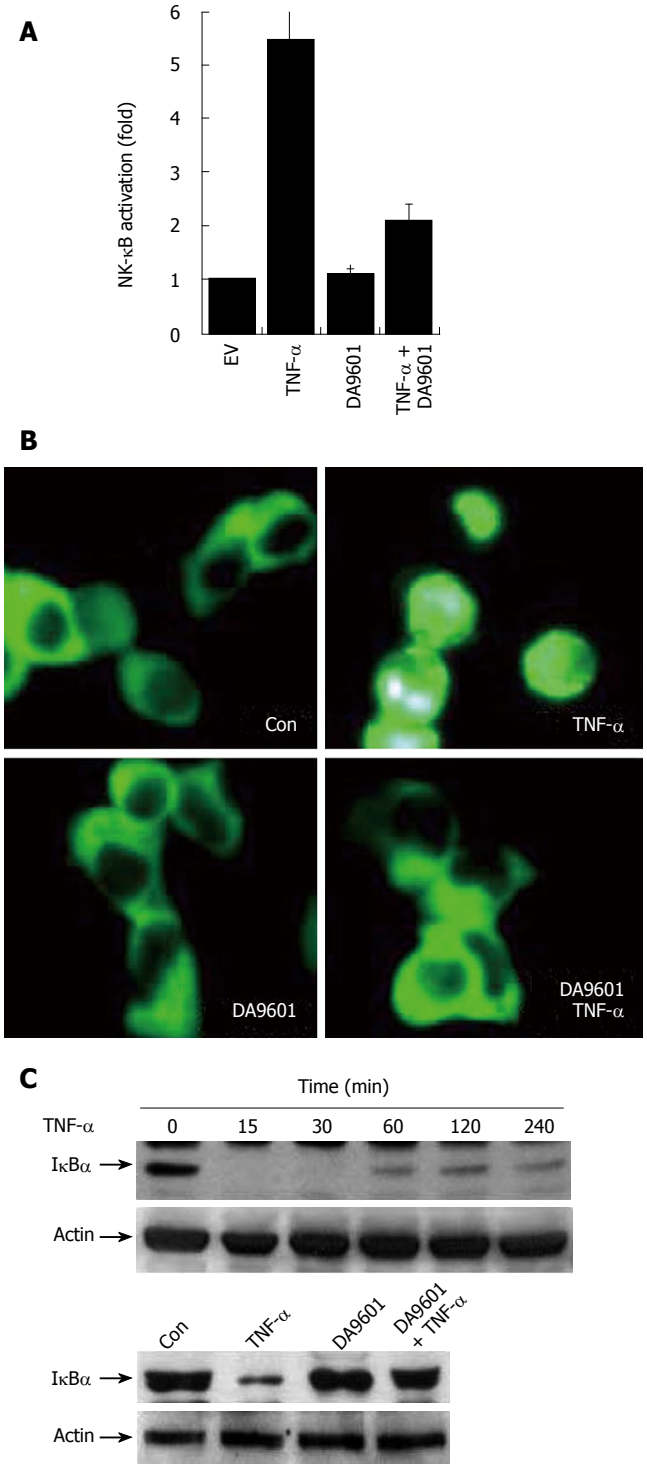


**Figure 4** DA-9601 blocks NF-κB activity in AGS cells. **A**: AGS cells (1 x $10^5$) were transfected with NF-κB luciferase reporter vector (0.8 μg/well). After 24 h of incubation, cells (1 x $10^5$) were pre-treated for 1 h with DA-9601 (50 μg/mL) and stimulated for additional 16 h with medium alone or medium containing TNF-α (5 ng/mL). At the end of incubation, cells were lysed, and the relative luciferase activity was measured using Luciferase Assay System; **B**: AGS cells (1 x $10^5$) were transfected with p65-EGFP vector (0.8 μg/well). After 24 h of incubation, cells were pre-treated for 1 h with DA-9601 (50 μg/mL) and stimulated for 1 h with medium alone or TNF-α. Nuclear translocation of p65-EGFP was observed under the fluorescence microscope (original magnification, 200 X); **C**: AGS cells (5 x $10^5$) were incubated with TNF-α (5 ng/mL) for the indicated time points (0-240 min) (top) or were pretreated with medium alone or with DA-9601 (50 μg/mL) for 1 h, and incubated with TNF-α for 30 min (bottom). The cell lysates were blotted with antibodies specific for the I-κBα and β-actin.

However, pre-incubation of these cells with DA-9601 (50 μg/mL) dramatically reduced TNF-α-induced promoter activities in both cell types, suggesting that DA-9601 inhibits IL-8 and CCL20 expressions and secretions *via* direct or indirect modulation of promoter activities.

### *DA-9601 inhibits TNF-α-induced NF-κB activity in both HEK293T cells and AGS cells*

Several recent studies have demonstrated that gene expression of both IL-8 and CCL20 is NF-κB dependent[18-21]. This led us to examine whether DA-9601 can inhibit NF-κB activity in TNF-α-treated AGS cells. We therefore examined the NF-κB activation by measuring NF-κB-dependent transcriptional activity, NF-κB p65 nuclear translocation, and I-κBα degradation. As shown in Figure 4A and B, incubation of AGS cells with DA-9601 for 24 h significantly decreased TNF-α-induced luciferase activity. We next measured nuclear translocation of the NF-κB p65 subunit. To this end, AGS cells were transfected with NF-κB-p65-EGFP vector for 24 h, and then the cells were further treated with DA-9601 (50 μg/mL), TNF-α (5 ng/mL), or DA-9601 (1 h before TNF-α treatment) plus TNF-α. As shown in Figure 3C, DA-9601 significantly inhibited nuclear translocation of NF-κB p65 subunit after 1 h of TNF-α treatment. We finally tested

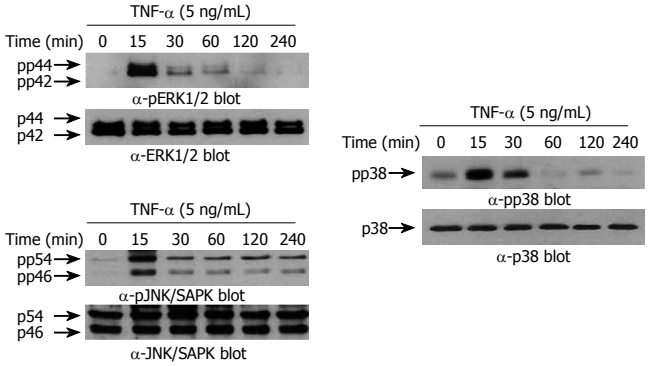


**Figure 5** TNF-α induces phosphorylation of MAPKs in AGS cells. AGS cells (5 x $10^5$ cells/well) were incubated for various times (0-240 min) with TNF-α (5 ng/mL). Protein extracts were prepared at the indicated time points, and then the levels of phosphorylated or total MAPKs (ERK-1/2 (*top*), p38 kinase (*middle*), and JNK/SAPK (*bottom*)) were determined by Western blotting using specific antibodies. The arrows indicate the position of specific immunoreactive bands corresponding to distinct MAPKs.

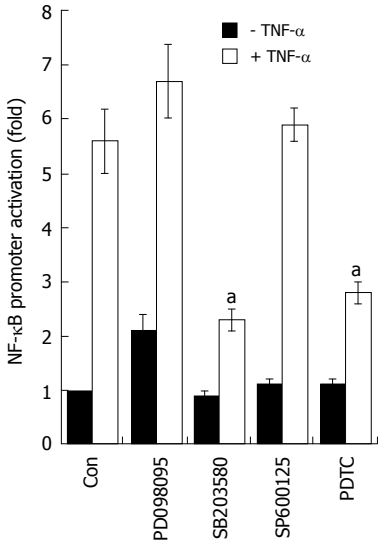


**Figure 6** SB203580 blocks NF-κB-dependent transcriptional activity in AGS cells. AGS cells (5 x $10^5$) were transfected with NF-κB luciferase reporter vector (0.8 μg/well). After 24 h of incubation, cells were pre-treated for 1 h with PD098059 (20 μmol/L), SB203580 (10 μmol/L), SP600125 (2 μmol/L), and PDTC (10 μmol/L); the cells were stimulated for additional 16 h with medium alone or medium containing TNF-α (5 ng/mL). At the end of incubation, cells were lysed, and the relative luciferase activity was measured using Luciferase Assay System. Note, $^aP < 0.05$, significantly different from control ($n = 4$).

whether DA-9601 inhibits I-κBα degradation in TNF-α-treated AGS cells. Treatment with TNF-α rapidly induced I-κB degradation (about 15 min) which later recovered slightly (> 240 min) (Figure 4C). However, pre-incubation of AGS cells with DA-9601 (1 h) significantly inhibited TNF-α-induced I-κBα degradation (Figure 4B). Taken together, we conclude that DA-9601 inhibits IL-8 and CCL20 expressions and their protein releases, presumably by acting at the site or upstream of NF-κB-dependent pathways.

### *p38 kinase plays a crucial role in TNF-α-induced NF-κB activity as well as CCL20 production in AGS cells*

Previous reports demonstrated that three structurally-related mitogen-activated protein kinases (MAPKs) play crucial roles in a variety of systems involving TNF-α[22-24]. We therefore asked whether TNF-α leads to phosphorylation of the MAPK subfamilies in AGS cells. As shown in Figure 5, treatment with TNF-α (5 ng/mL) rapidly induced phosphorylation of all three MAPK subfamilies. The maximal phosphorylation levels

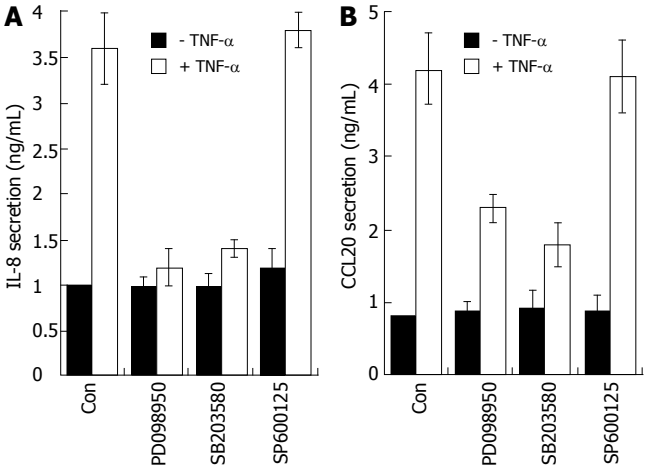


**Figure 7** Effects of MAPK modulators on IL-8 and CCL20 release by TNF-α in AGS cells. AGS cells (1 x $10^5$) were pre-treated for 1 h with or without selective MAPK inhibitors (PD098059, 20 μmol/L; SB203580, 10 μmol/L; SP600125, 2 μmol/L). The cells were then further incubated for 16 h with TNF-α (5 ng/mL). Levels of IL-8 and CCL20 protein were determined by ELISA. These data are representative of three independent experiments.

were achieved as early as 15 min in all MAPKs after TNF-α treatment, and thereafter the levels were gradually decreased. Interestingly, however, among three MAPK blockers used, the selective p38 kinase inhibitor SB203580 (10 μmol/L) could only inhibit TNF-α-induced NF-κB-dependent promoter activity (Figure 6). As expected, treatment with PDTC (10 μmol/L) also inhibited NF-κB-dependent promoter activity (Figure 6). These results suggest a functional cross-talk between p38 kinase and NF-κB-dependent signaling system, and further suggest that p38 kinase acts upstream of NF-κB activation, thereby inhibiting IL-8 and CCL20 promoter activities in AGS cells.

To further confirm that p38 kinase is involved in chemokine production, AGS cells were incubated with three MAPK inhibitors prior to TNF-α treatment and then the production of IL-8 and CCL20 was measured by the ELISA method. As expected, treatment with SB203580 significantly inhibited IL-8 and CCL20 production induced by TNF-α (Figure 7). The selective inhibitor of the MEK1 pathway PD098059 also inhibited IL-8 and CCL20 production in TNF-α-treated AGS cells, while it had no effect on NF-κB-dependent transcriptional activity (Figure 6). These results may suggest that, in terms of IL-8 or CCL20 production, ERK1/2 is not coupled with NF-κB-dependent pathways but may act at a post-transcriptional level.

### *DA-9601 inhibits p38 kinase phosphorylation, but shows little effect on ERK and JNK in AGS cells*

An important question raised at this point was whether DA-9601 also inhibits p38 kinase phosphorylation induced by TNF-α. To test this, AGS cells were treated with DA-9601 for 1 h, and then the cells were further incubated for 15 min with TNF-α (5 ng/mL). The phosphorylation levels of all three MAPKs were determined by Western blot analysis. Surprisingly, while having no effect on both ERK1/2 and JNK1/2, DA-9601 specifically and

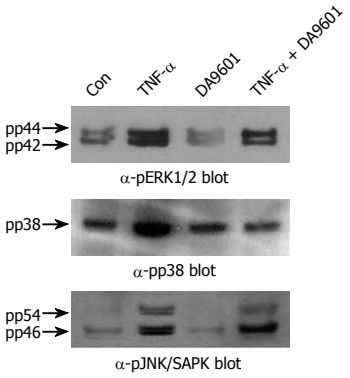


**Figure 8** DA-9601 selectively attenuates TNF-α-mediated phosphorylation of p38 kinase in AGS cells. Cells (5 x $10^5$ cells/well) were pretreated for 1 h with medium alone or medium containing DA-9601 (50 μg/mL). Then, the cells were stimulated for 15 min with or without TNF-α (5 ng/mL). The cell lysates were blotted with antibodies specific for the phosphorylated or total forms of ERK1/2, p38 kinase, and JNK/SAPK, and visualized using a peroxidase-conjugated secondary antibody and the ECL system.

significantly inhibited p38 kinase phosphorylation induced by TNF-α (Figure 8). These results strongly suggest that DA-9601 inhibits TNF-α-induced chemokine production as well as secretion via a direct or indirect inhibition of p38 kinase activity. Further, these results suggest that DA-9601 inhibits NF-κB-dependent transcriptional activity presumably by blocking the p38 kinase signaling system.

## DISCUSSION

In this study we analyzed the effect and mechanism of action whereby DA-9601 modulates the production of two chemokines; i.e., IL-8 and CCL20, in human gastric epithelial AGS cells. IL-8 is one of the key molecules that is involved in innate immunity, and is known to be elevated in gastric biopsy samples of patients with *H pylori*-associated gastritis[5]. In contrast, it was generally accepted that gastric epithelial cells do not produce the cytokines that are essential components of host adaptive immunity. However, recent study has shown that they do produce CCL20 upon infection with *H pylori*[9], thereby suggesting that CCL20 is also involved in gastric mucosal immunity. The present results demonstrate that AGS cells do produce both IL-8 and CCL20 chemokines in response to TNF-α stimulation. This evidence extends the current view and suggests that gastric epithelial cells may also have a critical function by inducing mucosal innate immunity as well as adaptive immunity.

The use of natural anti-inflammatory products provides an attractive and safe alternative to modulate inflammatory disorders. *A. asiatica* has been widely used for centuries in traditional Asian diets and medications without any serious side effects. Also, the standardized ethanol extract (DA-9601) of this medicinal plant has been shown to have strong antioxidative and anti-inflammatory effects in experimental animal models, such as esophageal mucosal damage[25,26], ethanol-induced gastritis[4], and cerulin-induced pancreatitis[1]. However, the mechanisms of action of DA-9601 *in vitro* and *in vivo* are still unclear. Our data indicate that TNF-α-mediated expression of chemokine genes in gastric epithelial cells is blocked by DA-9601 treatment. The mechanism of action of DA-9601 involves blockade of NF-κB activation, in agreement with previous studies using a mouse skin model[27]. To further define the mechanism by which NF-κB activity is inhibited by

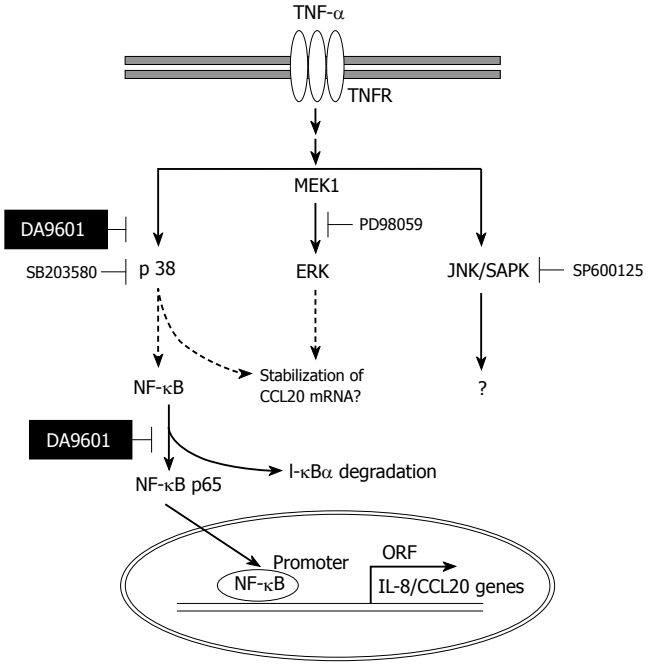


**Figure 9** Hypothetical mechanism of action of DA-9601 on TNF-α-induced CCL20 expression in AGS cells. TNF-α induces activation of three MAPKs. Among three MAPKs, however, activation of p38 kinase involves in NF-κB signaling system. DA-9601 may inhibit NF-κB directly or indirectly through the inhibition of p38 kinase pathway. See text for discussion.

DA-9601, we investigated molecular relationships between MAPKs and NF-κB. We found that SB203580, a selective inhibitor of p38 kinase, blocked NF-κB activity, thereby suggesting that p38 kinase may be functionally linked with NF-κB. In this regard, it is particularly interesting to note that DA-9601 could block activation of both p38 kinase and NF-κB in AGS cells. This suggests that DA-9601 does not directly block the NF-κB-dependent signaling system, but instead, it may indirectly inhibit NF-κB through the inhibition of p38 kinase pathways. Accordingly, several lines of evidence have suggested that p38 kinase lies upstream of NF-κB[28-30]. It is also interesting that PD098059, the upstream inhibitor of ERK1/2 pathway, had no effect on TNF-α-induced NF-κB activity, while it significantly blocked IL-8 and CCL20 production. These results suggests that the ERK1/2 pathway is not involved in the regulation of promoter activity but may participate in the stabilization of chemokine genes, as demonstrated by other reports[12,31].

Although DA-9601 has substantial anti-inflammatory or anti-oxidative effects, the structural identity of its active component(s) remains to be elucidated. Eupatilin, one of the major pharmacologically active ingredients of *A. asiatica,* may share its anti-inflammatory[32] or anti-oxidative effects[33] with DA-9601. However, our unpublished results demonstrated that eupatilin has no significant effect on TNF-α-induced IL-8 expression and secretion, while it has strong protective (anti-oxidative) effect for AGS cells from hydrogen peroxide-induced cellular damage (data not shown). This implies that DA-9601 may also have other active ingredient(s) that selectively inhibit(s) cytokine-induced expression or release of IL-8 and CCL20 proteins

as well as other inflammation-related proteins.

Collectively, the data obtained in the present study are compatible with the schematic representation in Figure 9. DA-9601 has an anti-inflammatory potential based on its blocking effects on TNF-α-induced IL-8 and CCL20 production. The inhibition by DA-9601 appears to be mediated through the inhibition of promoter activity as well as the NF-κB-dependent signaling system. Inhibition of p38 kinase by SB203580 blocked NF-κB activity, suggesting that p38 kinase is functionally linked with NF-κB and lies upstream of NF-κB. More interestingly, DA-9601 inhibited activation of both the p38 kinase and NF-κB-dependent systems. This suggests that DA-9601 inhibits NF-κB directly or indirectly through the inhibition of the p38 kinase pathway. Additional studies will be required to clarify the upstream signal transduction pathways that might be affected by DA-9601.

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**S- Editor** Wang J **L- Editor** Lutze M **E- Editor** Ma WH

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*World J Gastroenterol* 2006 August 14; 12(30): 4859-4865
***World Journal of Gastroenterology*** ISSN 1007-9327


*BASIC RESEARCH*

# Augmenter of liver regeneration promotes hepatocyte proliferation induced by Kupffer cells

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Received: 2006-03-16 Accepted: 2006-04-21

## Abstract

**AIM:** To observe the effects of augmenter of liver regeneration (ALR) on Kupffer cells and to determine whether ALR promotes hepatocyte proliferation induced by Kupffer cells.

**METHODS:** Kupffer cells and hepatocytes were cultured *in vitro* and various concentrations of recombinant rat ALR (rrALR) were added. $^3$H-thymidine, BrdU and $^3$H-leucine incorporation was determined in cultured Kupffer cells and hepatocytes, in hepatocytes conditioned by Kupffer cells, and in associated medium. rrALR was labeled by iodination and used to determine its binding activity by Scatchard analysis in Kupffer cells and primarily cultured rat hepatocytes.

**RESULTS:** rrALR stimulated DNA replication in Kupffer cells and protein synthesis both in cells and in medium in a non-concentration-dependent manner. The effect was significant at the concentration of 1 μg/L ALR. However, rrALR had no effect on primarily cultured hepatocytes, when hepatocytes were cultured with the Kupffer cell medium conditioned by ALR, DNA replication and protein synthesis in hepatocytes increased significantly at the concentration of 1 μg/L ALR. When the ALR concentration was increased, its effect on hepatocyte proliferation decreased to the basal level. Scatchard analysis indicated the presence of a single class of high affinity receptors with a dissociation constant (*Kd*) of 0.883 nmol/L and a maximum binding capacity (*Bmax*) of 126.1 pmol/g protein in the rat Kupffer cells.

**CONCLUSION:** ALR can promote hepatocyte proliferation induced by Kupffer cells, which is associated with the concentration of ALR, suggesting that Kupffer cells play a dual role in liver regeneration.



**Key words:** Liver regeneration; Augmenter of liver regeneration; Kupffer cell

Wang CP, Zhou L, Su SH, Chen Y, Lu YY, Wang F, Jia HJ, Feng YY, Yang YP. Augmenter of liver regeneration promotes hepatocyte proliferation induced by Kupffer cells. *World J Gastroenterol* 2006; 12(30): 4859-4865

http://www.wjgnet.com/1007-9327/12/4859.asp

## INTRODUCTION

Liver regeneration requires various factors promoting cellular proliferation. Among these, augmenter of liver regeneration (ALR)[1], a novel cytokine, similar to the hepatic stimulator substance[2], has been shown to be mainly involved in the process of liver regeneration. ALR was cloned by Hagiya *et al*[1] from the weanling rat liver in 1994. Subsequently, Giorda *et al*[3] and Yang *et al*[4] cloned the cDNA of human ALR. It was found that rat, mouse, and human ALR genes (and protein products) are highly conserved, homologous and preferentially expressed in testis and liver[3]. ALR and the Saccharomyces cerevisiae homologue Erv 1p are members of the new ALR/Erv 1 protein family[5-7]. Experiments and clinical research demonstrated that ALR could specifically promote hepatocyte proliferation and rescue liver failure[8-13]. The specific molecular impact of ALR on liver regeneration still remains unclear. Wang *et al*[14] have identified and characterized the receptor for human ALR on rat hepatocytes, and they found that human hepatoma cells *via* their specific receptor ALR may initiate a corresponding cytoplamic signal transduction. But it was also reported that ALR promotes liver regeneration by inhibiting the activity of hepatic natural killer (NK) cells[13] and rat ALR (rALR) has no effect on primary rat hepatocytes *in vitro*[15], suggesting that ALR may likely exert its biological effect by using hepatic non-parenchymal cells. Kupffer cells, the resident liver macrophages, have been implicated as the primary source of inflammatory factors but may also be the source of important growth

factors and cytokines that initiate cellular recovery and regeneration[16,17]. The protective role of Kupffer cells in hepatic injury by promoting liver regeneration has been recently emphasized[18-20], but the relationship between ALR and Kupffer cells in liver regeneration has not been investigated. Here we report the existence of ALR receptor in Kupffer cells and show the fact that ALR promotes hepatocyte proliferation induced by Kupffer cells.

## MATERIALS AND METHODS

### Isolation and primary culture of rat hepatocytes

Hepatocytes were isolated from male Sprague-Dawley rats (weighing 180-250 g, provided by Animal Center of Chinese Academy of Sciences) by *in situ* perfusion of the liver with collagenase (0.5 g/L type I CLSI, Sigma) as described previously[21]. The viability of the cells was determined by trypan blue exclusion. Preparations with their viability greater than 85% or higher were used. The cells were suspended in Williams' medium E (Sigma) containing 100 mL/L fetal calf serum, 2 mmol/L L-glutamine, 7 mg/L insulin and 10 mmol/L HEPES, plated in 24-well plates ($0.125 \times 10^6$ cells/ well), and incubated in an atmosphere of 95% air and 5% $CO_2$ at 37℃. The medium was renewed after incubated for 3 h and cells were used for experiments after an overnight incubation.

### Preparation of Kupffer cells

Kupffer cells were prepared from the livers of male Sprague-Dawley rats (200-224 g) as described previously[22]. Briefly, following the collagenase (type Ⅳ from 0.25 g/L clostridum histoticum, Sigma)/protease (type XIV from 0.5 g/L streptomyces griseus, Sigma) digestion of liver and removal of hepatocytes and cell debris by low speed centrifugation, Kupffer cells and endothelial cells were purified from other non-parenchymal cells by density gradient centrifugation using 300 g/L metrizamide. Finally Kupffer cells were isolated by a centrifugal elutriation procedure[22]. The viability of Kupffer cell preparation was greater than 95% as determined by trypan blue exclusion. Kupffer cells were suspended in Williams' medium E containing 100 mL/L heat-inactivated fetal calf serum, 5 MU/L penicillin and 5 g/L streptomycin and plated in 24-well culture plates at a density of $1 \times 10^6$ cells/well, then plated in 48-well culture plates at a density of $0.5 \times 10^6$ cells/well and incubated in an atmosphere of 95% air and 5% $CO_2$ at 37℃. The medium was renewed after incubated for 3 h and cells were used for experiments after an overnight incubation.

### Determination of the effects of Kupffer cells-conditioned medium on hepatocytes

Kupffer cells were washed and placed in serum-free Williams' medium E containing 0.1% bovine serum albumin (BSA, Sigma) and the test ALR concentrations (control, 1, 10, 100 and 1000 μg/L). After 24 h, the medium was filtered (0.2 μm, Gibco) and transferred to the cultured hepatocytes. Hepatocytes were incubated for 24 h with the test agents, the medium was conditioned by Kupffer cells, and various determinations were then made.

### $^3$H-thymidine incorporation assay: Determination of DNA replication

Kupffer cells or hepatocytes were rinsed twice with Hanks' buffered saline solution (HBSS), and placed in serum-free Williams' medium E containing 1 g/L BSA and the test ALR concentrations (control, 1, 10, 100 and 1000 μg/L) for 24 h. After the medium was aspirated from Kupffer cells or hepatocytes, a fresh medium containing 37 MBq/L $^3$H-thymidine (Amersham Pharmacia Biotech, USA) was added to the culture wells. Following an incubation of 4 h at 37℃, the cells were washed with ice-cold HBSS containing 0.1% BSA, treated with 10% ice-cold trichloroacetic acid (TCA) for 10 min, and washed once with TCA followed by 95% ethanol. The cells were then digested with 5% sodium dodecyl sulfate (SDS) at 60℃ for 25 min. The liquid was transferred into scintillation vial, and washed once more with 0.5 mL 0.5% SDS. The radioactivity was determined using 5 mL β-scintillation solution.

### $^3$H-leucine incorporation assay: Determination of protein synthesis

Kupffer cells or hepatocytes were stimulated for 24 h as in the $^3$H-thymidine incorporation assay. After the medium was aspirated, a fresh medium containing 37 MBq/L $^3$H-leucine (Amersham Pharmacia Biotech, USA) was added to the culture wells. Following an incubation of 4 h at 37℃, the medium aspirated from Kupffer cells or hepatocytes was transferred to 1.5 mL Eppendorf tubes, BSA (finally 5%) and TCA (finally 5%) were added for 15 min, centrifuged at 12900 r/min for 20 min at 4℃, washed three times with 5% ice-cold TCA. Then 200 μL 0.1mol/L NaOH was added to dissolve the pellet, pH was adjusted with 0.1 mol/L HCl, the liquid was transferred to the scintillation vial. The cells were washed with ice-cold HBSS containing 1 g/L BSA, treated with 100 g/L ice-cold TCA for 10 min, and washed once with TCA followed by 95% ethanol. The cells were then digested with 5% SDS at 60℃ for 25 min. The liquid was transferred to scintillation vial, and washed once with 0.5 mL 0.5% SDS, the radioactivity was determined using 5 mL β-scintillation solution.

### BrdU incorporation assay

Cells were plated in 12-well plates, serum-starved, and stimulated as described previously in the $^3$H-thymidine incorporation assay. After 24 h of stimulation, the cultures were incubated with BrdU labeling reagent (Dako) diluted at 1:1000. Cells were rinsed and fixed for 30 min at room temperature with acetic alcohol (90% ethanol, 50 mL/L acetic acid, 50 mL/L distilled $H_2O$). Endogenous peroxidase was blocked by incubation in 1% $H_2O_2$ in methanol for 20 min at room temperature. Cells were washed in PBS and incubated for 15 min in 1.5 mol/L HCl at 37℃, and extensively washed in PBS. Cells were then incubated in anti-BrdU antibody (mouse IgG, Sigma) diluted at 1:40 in PBS containing 1% BSA for 1 h at 37℃ and washed in PBS. Cells were incubated in anti-mouse secondary antibody (goat anti-mouse IgG, Sigma) diluted

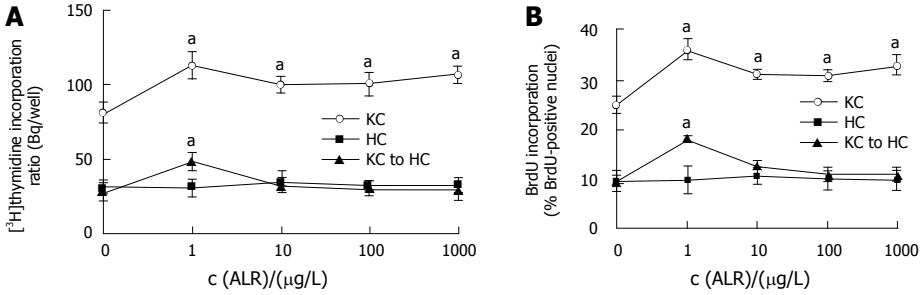


**Figure 1** Effects of ALR on proliferation of Kupffer cells and hepatocytes. **A**: DNA replication; **B**: BrdU-positive nuclei. KC: Kupffer cells; HC: Hepatocytes; KC to HC: Hepatocytes conditioned by Kupffer cells. $^{a}P < 0.05$ *vs* control, $n = 3$.

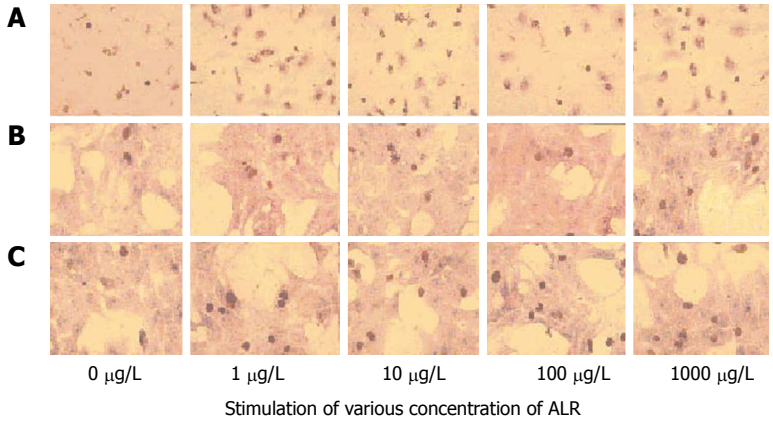


**Figure 2** BrdU immunohistochemistry. The nuclei of BrdU-positive cells were stained brown. **A**: Kupffer cells; **B**: Hepatocytes; **C**: Hepatocytes conditioned by Kupffer cells (× 100).

at 1:100 in PBS, and 10 g/L BSA for 30 min at room temperature followed by washing. Cells were incubated in ABC solutions for 30 min at room temperature, washed and incubated in 50 mmol/L Tris (pH 7.6) for 5 min, then the 3, 3-diaminobenzidine solution was added. Three areas in each well were counted for a total of about 1000 cells. Proliferation was indicated as a percentage of labeled nuclei.

### $^{125}$I- recombinant rat ALR binding to cultured kupffer cells and primary hepatocytes

Recombinant rat ALR (rrALR) was expressed in *Escherichia coli* and prepared with high purity (> 95%) as described previously[23]. rrALR was radioiodinated with the chloramines-T methods[14,24]. Briefly, 15 uL of 50 mmol/L sodium phosphate buffers (pH7.0) and 18.5 MBq/L sodium -$^{125}$I (Amersham Pharmacia Biotech, USA) were added to a siliconized tube containing 5 μg of rrALR. The reaction was started by adding 10 μL of chloramine -T reaction using 20 μL of ending solution (50 nmol/L N-acetyl-L-tyrosine, 0.01 mol/L sodium phosphate buffer). $^{125}$I-rrALR was separated from free iodine by gel filtration on a column (20 cm× 11.0 cm) of Sephadex G-25 (Amersham Pharmacia Biotech, USA) equilibrated with PBS and 1 g/L BSA. The fractions containing $^{125}$I-rrALR were pooled. The assay was performed as described previously[14, 23]. Kupffer cells and rat hepatocytes were washed in HBSS (containing 20 mmol/L HEPES, 1.3 mmol/L $CaCl_2$, 0.5 mmol/L $MgCl_2$, 1% BSA, pH 7.0), and pre-incubated in the presence of the same buffer for 30 min at 25℃. After equilibration was achieved in this medium, 0.3 mL containing 12.5 pmol/L-3200 pmol/L $^{125}$I-rrALR with or without 5 μmol/L unlabeled rrALR( saturation binding) was incubated for 3 h at 25℃ with constant shaking. The cells were washed 4 times with ice-cold PBS and digested with 0.5 mL 0.75 mol/L NaOH for 30 min at 37℃, and the radioactivity in the digest was determined in a gamma-counter (cpm). Specific binding of $^{125}$I-rrALR was calculated as the difference between cell-associated radioactivity in the presence and absence of unlabeled rrALR.

### Statistical analysis

The values are presented as mean ± SD of triplicate determinations. Each experiment was repeated at least three times. Student's *t*-test was employed for statistical comparison of the paired samples. $P < 0.05$ was considered statistically significant.

## RESULTS

### Effects of ALR concentration on Kupffer cells

$^{3}$H-thymidine incorporation assay showed that rrALR stimulated DNA replication in Kupffer cells in a non-concentration-dependent manner (Figure 1A). The significant effect was observed at the concentration of 1 μg/L ALR, the DNA replication increased to 144.2% as compared to the control (117 ± 11.7 Bq/well *vs* 81.2 ± 6.7 Bq/well, $P < 0.05$). Following the increase of added ALR concentration, DNA replication began to decrease, but was still higher than that in the control ($P < 0.05$). Being consistent with the $^{3}$H-thymidine incorporation assay, cell labeling by BrdU increased to 36% from a basal level of 24% at the concentration of 1 μg/L ALR ($P < 0.05$, Figure 1B, Figure2). Similar to the DNA replication, ALR also produced a non-concentration-dependent transforming of protein synthesis both in Kupffer cells and in associated medium, but the significant effect was observed at the

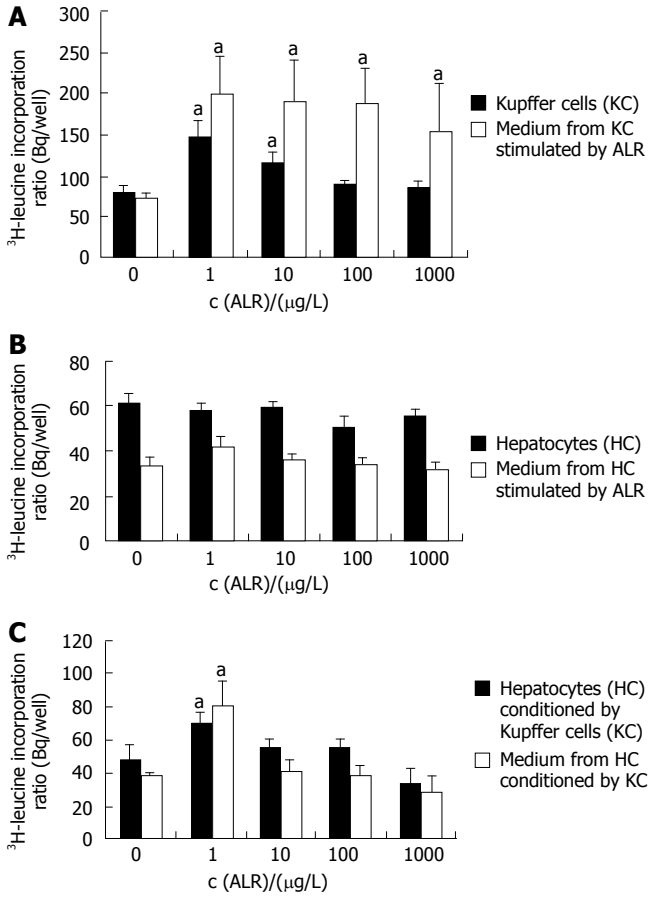


**Figure 3** $^3$H-leucine incorporation assay. **A**: Kupffer cells and associated medium; **B**: Hepatocytes and associated medium; **C**: Hepatocytes conditioned by Kupffer cells and associated medium. $^aP < 0.05$ *vs* control, $n = 3$.

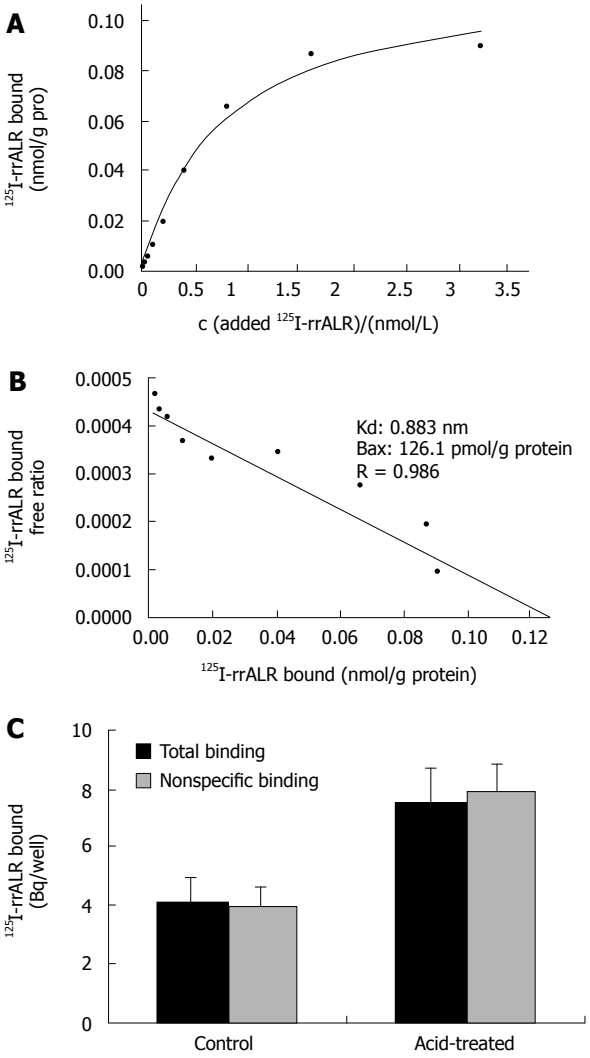


**Figure 4** $^{125}$I-rrALR binding assay. **A**: Saturation cures of $^{125}$I-rrALR binding to its receptor on Kupffer cells; **B**: Scatchard plot of the ALR receptor on Kupffer cells; **C**: Binding of ALR to cultured hepatocytes (mean ± SD, $n = 3$).

lowest concentration of 1 μg/L ALR tested (Figure 3A). The effects in cells and associated medium elevated to 182.6% and 275.3%, respectively, as compared to those in the control (cells:147.5 ± 19.6 Bq/ well *vs* 80.8 ± 5.9 Bq /well, $P < 0.05$; associated medium: 198.0 ± 46.1 Bq / well *vs* 71.9 ± 7.5 Bq/well, $P < 0.05$). Following the increase of added ALR concentration, protein synthesis in associated medium decreased, but was still higher than that in the control ($P < 0.05$, Figure 3A).

### *Effects of ALR concentration on hepatocytes*

ALR did not cause any significant change in DNA replication and protein synthesis in hepatocytes or in associated medium (Figures 1A and B, Figure 2, Figure 3B).

### *Effects of the medium conditioned by Kupffer cells with ALR on hepatocyte proliferation*

Stimulation of hepatocytes with the medium conditioned by Kupffer cells with ALR increased DNA replication and protein synthesis at the lowest concentration of 1 μg/L ALR tested, to 174.3% ($^3$H-thymidine incorporation, Figure1A) or 180% (BrdU labeling, Figure1B, Figure 2), 146.2% (in cells) and 210.7% (in associated medium) (Figure 3C) respectively, as compared to that in the control (DNA replication: 48.4 ± 3.5 Bq/ well *vs* 27.8 ± 4.7Bq/well in $^3$H-thymidine incorporation, 18.0% vs !0.0% in BrdU labeling; protein synthesis:70.5 ± 5.7 Bq/ well vs 48.2 ± 9.2 Bq/well in cells, 80.7 ± 15.0 Bq/well *vs* 38.3 ± 2.5 Bq/well in associated medium, $P < 0.05$). But with the increase of added ALR concentration, the medium conditioned by Kupffer cells with ALR produced a concentration-dependent inhibition of DNA or protein synthesis in hepatocytes, the significant effect was observed at the highest concentration of 1000 μg/L ALR tested (DNA: 28.9 ± 5.8 Bq /well, Figure 1A; protein synthesis: 33.9 ± 9.0 Bq/well in cells, 29.3 ± 8.9 Bq /well in the medium, Figure 3C).

### *$^{125}$I-rrALR binding assay*

Typical saturation curves of $^{125}$I-rrALR binding to cultured Kupffer cells are shown in Figure 4A. Specific binding of ALR was saturated at about 1.5 nmol/L. Scatchard analysis resulted in a rectilinear plot (Figure 4B), thereby suggesting the presence of a single class of high affinity binding sites, namely the existence of a receptor of ALR on Kupffer cells. The $^{125}$I-rrALR binding capacity to cultured Kupffer cells was 126.1 ± 22.3 pmol/g protein, the affinity of the receptor was 0.883 ± 0.056 nmol/L.

However, similar to Gandhi *et al*[23], no receptor for

ALR was found on hepatocytes by radioligand binding analysis. To investigate the possibility that ALR receptors on hepatocytes are down-regulated, the acidic medium, a procedure known to dissociate bound peptide ligand from its receptors, was used to treat cultured hepatocytes as previously described[21,22], but it did not unmask any specific binding of ALR yet (Figure 4C).

## DISCUSSION

Previous studies have revealed that ALR appears to be an important regulator of liver regeneration. Recently, emphasis has been placed on ALR molecular mechanisms underlying liver regeneration. Many advances have been gained in murine and human experiments. For instance, Yang *et al*[4] have cloned the cDNA of human ALR (hALR) from human fetal liver lysates encoding 125 amino acids and 15KD in molecular weight. hALR could stimulate proliferation of cultured hepatocytes as well as hepatoma cells *in vitro* and rescue acute hepatic failure *in vivo*. ALR exerts its hepatrophic activities through paracrine and autocrine pathways. Wang *et al*[14] have demonstrated the existence of hALR specific receptor on the surface of primary hepatocytes and hepatoma cells. Through the ALR receptor, ALR stimulates hepatocyte proliferation by activating the mitogen-activated protein kinase cascade (MAPK signaling pathway)[25]. The existence of ALR in nuclei and cytosol of liver tissues further implicates that ALR has intracellular function in hepatocytes[26]. In immunohistochemical studies, Thasler *et al*[27] found that hALR is mainly expressed both in normal and in impaired hepatocytes. Even though there exist many inconsistent issues, Gandhi *et al*[23] have failed to show any specific rALR receptor in hepatocytes. Hagiya *et al*[1] and Yu *et al*[12] reported that rat ALR has no effect on primary rat hepatocytes *in vitro* and that the expression of ALR is absent in normal hepatic tissues[15]. The existence of contrary results together with the mechanism of inhibiting hepatic NK activity to promote liver regeneration suggests that it is possible for ALR to exert its bioactivities by interacting with other cytokines or with hepatic non-parenchymal cells on hepatocytes.

In this study, we showed the presence of high affinity receptors of ALR on hepatic Kupffer cells, and found that ALR could stimulate proliferation of Kupffer cells, suggesting that ALR stimulates hepatocyte proliferation by activating Kupffer cells, which may improve our understanding of the molecular mechanism of the biological action of ALR and its effect on liver regeneration. $^3$H-thymidine and $^3$H-leucine incorporation assay and BrdU labeling demonstrated that ALR stimulated DNA and protein synthesis of Kupffer cells in a non-concentration-dependent manner and had no effect on primary hepatocytes. But with the medium conditioned by Kupffer cells, ALR promoted hepatocyte proliferation. Similar to this study, Chen *et al*[28] reported that when hepatocytes are co-cultured with Kupffer cell supernatant only activated by LPS, hepatocyte DNA and protein synthesis are increased significantly. Recently Armburst *et al*[19] reported that the early gene expression of hepatocyte growth factor in Kupffer cells after carbon tetrachloride ($CCl_4$) treatment may be an important event in the early phase of liver regeneration. Together with the finding about the entry of ALR into Kupffer cells by immunolabelling[23], these events suggest that the medium containing some kind of hepatocyte growth factors coming from Kupffer cells conditioned by ALR and secreting into the medium, can regulate hepatocyte proliferation. The increased $^3$H-leucine incorporation showed the protein synthesis both in Kupffer cells and in associated medium. What kind of protein is needed should be further studied. The foregoing studies have tested acute reactant cytokines such as interleukin-6 (IL-6) and tumor necrosis factor (TNF)-α originated mainly from activated Kupffer cells which are the chief growth factors in hepatocyte proliferation *in vivo*[17,29-33]. Since TNF-α and IL-6 are involved in the initiation of liver regeneration, we assume that ALR, like LPS, may activate Kupffer cells by unknown means and then mediate Kupffer cell-dependent release of TNF-α and IL-6, triggering hepatocyte proliferation. The ALR receptor on Kupffer cells can elucidate the effect of ALR on Kupffer cells in part. Here, the high affinity receptor for ALR on Kupffer cells can be identified by $^{125}$I-ALR binding assay. When ALR binds to the cell surface receptor, Kupffer cells are activated and then release various cytokines initiating liver regeneration. The binding effect is saturable. When an excess amount of unlabeled ALR is added, it could replace the cell surface sites to which ALR has bound. It was reported that $^{125}$I-ALR is found to have the similar molecular weight and biological activity as the unlabeled ALR, and that iodination does not change the characteristics of natural ALR, suggesting that $^{125}$I-ALR can be used for identification of ALR receptor[14]. As for the specificity of this binding, further researches need to be done. In our study, as the concentration of ALR was added to 1000 μg/L, the effect of ALR-dependent-Kupffer cells on hepatocyte proliferation was inhibited, suggesting that Kupffer cells have a dual function as a regulator in liver regeneration. Responding to the immoderate proliferation effect of hepatocytes, Kupffer cells exert their inhibitory effects by releasing growth inhibiting factors. Transforming growth factor(TGF)-β is the most obvious candidate because it has been shown *in vivo* models that TGF-β could antagonize TNF-α actions and induce apoptosis, constraining the amount of hepatocyte proliferation[34-36].

Similar to the study by Gandhi *et al*[23], no receptor for rALR was found on hepatocytes in our study, which may interpret the inability of rALR to exert a mitogenic effect on hepatocytes *in vitro*. To investigate the possibility that ALR receptors on hepatocytes are down-regulated, the acidic medium, a procedure known to dissociate bound peptide ligand from its receptors was used to treat cultured hepatocytes, but it did not unmask any specific binding of ALR. At the same time, there is evidence that hepatocytes synthesize and secrete ALR[23,37]. A further finding showed that hepatic ALR levels decreased while circulating ALR levels increased 12 h after 70% hepatectomy, suggesting that the release of stored ALR from hepatocytes rather than the accelerated synthesis increases the circulating

ALR level[28].

How should these events be put together? We hypothesize that under pathological or biological circumstances of liver regeneration such as partial hepatectomy or weaning livers, activated Kupffer cells release various cytokines initiating liver regeneration, and that the receptor of ALR on hepatocytes (assuming its existence) and ALR which are constitutively expressed and stored in hepatocytes in an inactive form beforehand, are released from the cells in an active form by unknown means, then ALR exerts its hepatrophic activities through paracrine and autocrine pathways such as binding to Kupffer cells or the inhibition of hepatic NK cells to promote hepatocyte proliferation. To prevent excess hepatocyte proliferation, anti-hepatotrophic growth factors such as TGF-β are produced.

In conclusion, the mechanisms of liver regeneration are involved in complicated interactions of various cytokines and cells[29,37]. Kupffer cells contain high affinity receptors for ALR on cell surfaces, by which ALR promotes hepatocyte proliferation. Further studies focusing on the examination of the specificity of receptors on Kupffer cells are needed.

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**S- Editor** Pan BR **L- Editor** Wang XL **E- Editor** Ma WH

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*World J Gastroenterol* 2006 August 14; 12(30): 4866-4869
***World Journal of Gastroenterology*** ISSN 1007-9327


*BASIC RESEARCH*

# Differentiation of hepatocytoid cell induced from whole-bone-marrow method isolated rat myeloid mesenchymal stem cells

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Received: 2006-04-13 Accepted: 2006-05-25

## Abstract

**AIM:** To explore the expansion and differentiation of hepatocytoid cell induced from myeloid mesenchymal stem cell (MSC) *in vitro*, in order to find suitable resource of hepatocytes for bioartificial liver or liver transplantation.

**METHODS:** The rat myeloid MSC was isolated and divided into three groups which were cultured by Friedensteion method, and then were induced by culture fluid, culture fluid plus cholestatic serum and culture fluid plus hepatocyte growth factor (HGF), respectively. Hepatocytoid cell as well as expression of CK18 and AFP was observed by immunohistochemistry.

**RESULTS:** After the induction for 21 d, hepatocytoid cell was observed, and its expression of CK18 and AFP was detected by immunohistochemistry in MSC cultured with cholestatic serum. Furthermore, on the 35$^{th}$ d, albumin mRNA was expressed in the cell, suggesting the inducing effect was similar to that by HGF.

**CONCLUSION:** Rat myeloid MSC can differentiate into hepatocyte lineage under appropriate condition. This method is easy to operate.



**Key words:** Mesenchyme stem cell; Hepatocytoid cell; Expansion; Differentiation; Induction

Li W, Liu SN, Luo DD, Zhao L, Zeng LL, Zhang SL, Li SL. Differentiation of hepatocytoid cell induced from whole-bone-marrow method isolated rat myeloid mesenchymal stem cells. *World J Gastroenterol* 2006; 12(30): 4866-4869

http://www.wjgnet.com/1007-9327/12/4866.asp

## INTRODUCTION

Over the past few years, great progress has been made in the research of stem cell. It has been proved that some stem cells in bone marrow can differentiate into various kinds of cells. Therefore, through culturing myeloid stem cells *in vitro*, and providing suitable conditions, these cells can be induced to generate and differentiate into other cells. There have been some reports that application of cytokines such as hepatocyte growth factor (HGF), fibroblast growth factor-4 (FGF-4) might promote myeloid stem cells to proliferate and differentiate into hepatocytoid cell. However, the cytokines are costly. This study was to explore a convenient way to induce myeloid mesenchyme stem cell (MSC) into hepatocytoid cell *in vitro*.

## MATERIALS AND METHODS

### Materials

One-month-old Wista rats were provided by the Animal Center of Tongji Medical College, Huazhong University of Science and Technology. Rat recombinant hepatocyte growth factor (HGF) (R&D Co.), Dulbecco's minimum essential medium (DMEM) (Gibco), fetal bovine serum(FBS, Hyclone), N-2-hydroxyethylpiperazine-N'-2'-ethanesulfonic acid (HEPES), galactose solution of insulin, transferrin and selenite(ITS) (Sigma), micotinamide, trypsinase(Gibco), proline(Wuhan Lingfei Bio. Co.), alpha fetoprotein(AFP) monoclonal antibody (MoAb) (Santa Cruz Co.), cytokinin 18 (CK18) MoAb, biotin labeled Ab, diaminobenzidine(DAB), streptavidin peroxidase (S-P) (Beijing Zhongshan Co.), deoxy-ribonucleoside triphosphate (dNTPs), Pyrotest Taq enzyme(Bao Bio Ltd. Co.), Bio-11-dUTP (Huamei Biotech Co), Deion formamide, salmon sperm DNA(Sigma), fluorescein isothiocyanate (FITC) labeled antibiotin(Boshide) and albumin primer 5'ATACACCCAGAAAGCACCTC3', 5' CACGAATTGTGCGAAGTC AC3' (Shenggong Co) were prepared.

### Methods

**Isolation and culture of myeloid MSC:** According to the literature[1], rat femurs were separated under aseptic condition. The myeloid cell, after douched and blew out from the bone with culture fluid (5 mL DMEM + 10% FBS), was added to the culture fluid to make cell suspension. Then the culturing was begun with the supplementation of fluid of DMEM, 10% FBS, 100 U/

mL penicillin and 100 U/mL streptomycin, at 37℃, in 50 mL/L CO2 environment. On the third day non-adherent cell was removed and the remaining cells were allowed to grow. On the 10th d the confluent cells were trypsinized and passaged. When the laminating cell with typical cellular appearance was observed, 0.25% trypase was added for digestion for 5-10 min and then the whole medium was added to terminate the digestion. When the innoculation density was $1 \times 10^4/cm^2$, the nutrient medium was added and the serial subcultivation was begun. The above procedure was repeated to passage for every 3-4 d.

**MSC differentiation potency detection:** The third generation MSC was inoculated onto 6-well culture plate (density: $5 \times 10^4$/mL), and the induction culture fluid (0.05 g/L vitamin C, 40 ng/mL dexamethasone, 10 mmol/L β sodium glycerophosphate) was added. Then the cell was passaged for every 4 d. On the 15th d autoclaved coverslips were put into the media and 3 d later the coverslips were taken out and were stained by alkaline phosphatase.

**Cholestatic serum preparation:** Male rats with weight of 400 g were anesthetized with Pentothal and their abdomens were degermed. After the abdominal cavities were opened, their choledochuses were ligated and then the bellies were sutured. Given normal feed for 10 d, those rats were dissected again at abdomen to obtain blood from inferior caval vein. The serum was separated and preserved in refrigerator at -40℃.

**Group division:** Three groups, including MSC culture group (only with culture fluid), MSC cultured with cholestatic serum (5%) group and MSC cultured with HGF (0.5 mg/mL) group, were divided. The culture fluid composed of DMEM, 10% FBS, 15 mmol/L HEPES, 10 mmol/L niacinamide, 1 mmol/L vitamin C, $10^{-7}$ mol/L dexamethasone, 1 mg/mL galactose, 30 μg/mL proline, ITS and antibiotics. All the cells were inoculated onto 6 well culture plate and allowed to creep on the plate coverslips custodited with polylysine (0.025%). The morphology of the cell was observed on the 7th, 14th, 21st and 35th d according to the literature [1] and during those days the coverslips were taken out and fixed with paraform (4%) for 30 min.

**Detection methods:** The expression of CK18 and AFP by the cells was detected by immunohistochemistry and S-P method, with PBS instead of 1st-antibody in negative control. The albumin expression was detected by *in situ* hybridization. Biotin random primer labeling method was applied as albumin labeling probe.

## RESULTS

### *Observation of cell growth condition*

**Growth condition of MSC culture group:** The primary cells adhered to the wall after cultured for 48 h, and the adhered cells increased gradually afterward. After 3 passages, on the 14th d, the cells showed uniform morphology (Figure 1) like fibroblast and grew as grass bunch or whirlpool.

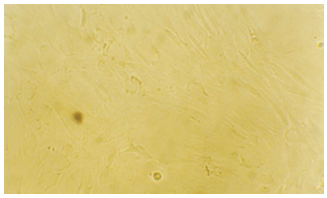

**Figure 1** After 3 passages, on the 14th d, the cell showed uniform morphology: fusiform shape or polygon shape, like fibroblast and grew as grass bunch or whirlpool (100 ×).

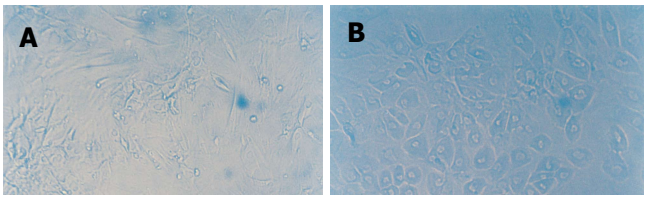


**Figure 2** Growth condition of MSC cultured with cholestatic serum. **A**: Cultured with cholestatic serum for 7 d, peripheral cell of the clone showed cuboidal morphology, which is characteristic of hepatocytes (100×); **B**: Cultured with cholestatic serum for 35 d, all MSCs showed cuboidal morphology, which is characteristic of hepatocytes (100×).

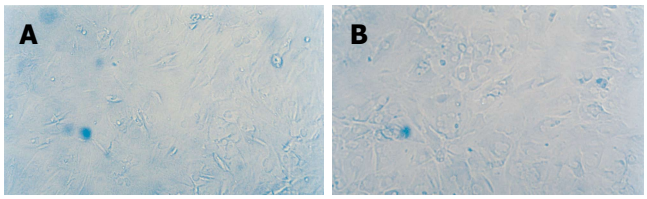


**Figure 3** Growth condition of MSC cultured with HGF. **A**: Cultured with HGF for 7 d, peripheral cell of the clone showed cuboidal morphology, which is characteristic of hepatocytes(100×); **B**: Cultured with HGF for 35 d, all MSCs showed cuboidal morphology, which is characteristic of hepatocytes (100×).

**Growth condition of MSC cultured with cholestatic serum group:** From the 35th d, the cell shape changed from fusiform to polygon and the proportion of the polygon cell increased with time. Meanwhile, the cell proliferation was also slowed. These phenomena appeared first in the periphery of the cell clone, and gradually extended into the whole cell clone (Figure 2).

**Growth condition of MSC cultured with HGF group:** The characteristics of the cell in this group were similar to that in MSC cultured with cholestatic serum group (Figure 3).

### *MSC identification*

After induced by vitamin C, dexamethasone and β sodium glycerophosphate, respectively, the cells gradually changed to different morphological forms, like triangle, fusiform and polygon. With the prolongation of culturing time, the cell population increased and cell volume enlarged. On the 15th d, the cell endochylema was full and the cells connected with each other with ecphyma. Alkaline phosphatase staining showed positive (Figure 4). This verified that the cells isolated were MSCs.

### *Expression of CK18 and AFP*

Expression of AFP by the cell was observed on the 14th d and expression of CK18 was detected on the 21st d in MSC cultured with cholestatic serum. And these two

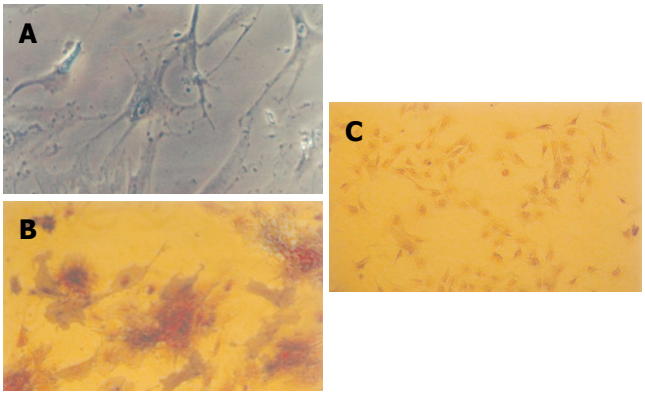


**Figure 4** MSC identification. **A**: Induced by vitamin C, dexamethasone and β sodium glycerophosphate, MSC differentiated into osteoblast (200×); **B**: Alkaline phosphatase stain showed positive (400×); **C**: Alkaline phosphatase stain negative control (100×).

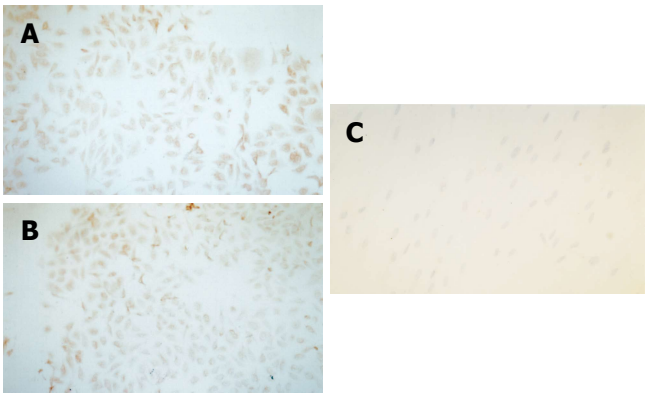


**Figure 5** Expression of CK18 and AFP. **A**: Cultured with cholestatic serum for 14 d, MSC expressed AFP by S-P method (100×); **B**: Cultured with HGF for 14 d, MSC expressed CK18 by S-P method (100×); **C**: Negative control (100×).

manifestations could still be observed on the 35th d. As comparison, MSC cultured with HGF also showed the same distinction but the other groups did not show the feature (Figure 5).

### *Expression of albumin*

Albumin was expressed both in MSC cultured with cholestatic serum and MSC cultured with HGF groups, but the former was more significant (Figure 6).

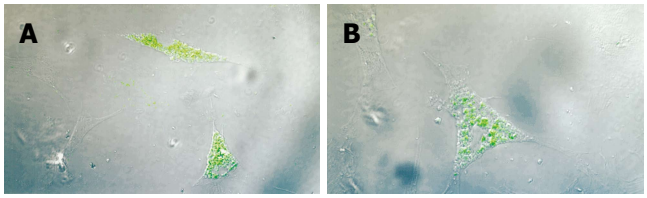


**Figure 6** Expression of albumin. **A**: Culture with cholestatic serum for 35 d, expression of albumin was confirmed by in situ hybridization (FITC dyeing ) (400 ×); **B**: Culture with HGF for 35 d, expression of albumin was confirmed by in situ hybridization (FITC dyeing ) (400×).

## DISCUSSION

There are two approaches to isolate MSCs including whole-bone marrow method [2] and density gradient centrifugation method[3]. The former, by which we employed in this experiment, is to purify MSCs by removing the non-attached cells when culture fluid is changed, according to the different adhering capabilities of stem cell. The latter is by adherently culturing mononuclear cell, based on the conception that different cell component in myeloid has different density. Nowadays, some new techniques have been developed to isolate MSCs, such as flow cytometry and immunomagnetic beads method. However, those new methods, being costly and technically difficult, have defect of inhibiting proliferation after sorting. So we preferred the whole-bone marrow method to purify MSC in this experiment, although the cells were of inadequate uniform, and heterogeneous in population.

Up to now, there has been no agreement on the phenotypical characterization of a "pure" population of human MSC despite the panoply of surface antigens reported to be expressed on MSC[4-6]. Therefore, identification of MSC has been a puzzle. In 1999, Pittenger *et al*[4] isolated MSC from human flank bone marrow and proved that MSC is pluripotent stem cell in that it could be induced into osteoblast, chondrocyte and lipocyte. Thus, through analyzing the differentiation phenotype during culturing, we could identify MSC. In our experiment, it was observed that osteoblast cytoplasm was basophilic and AKP stain positive. Because the osteoblast was the unique cell in differentiated MSC population, it proved the existence of MSC.

Recently, Verfaillie *et al*[7] demonstrated that multipotent adult progenitor cells(MAPCs) from rat, mouse and human could differentiate into functional hepatocytes. They detected that the cells could synthesize urea, albumin and amylon, possess activity of cytochrome P450, adsorb α-low-density lipoprotein and did not have carcinogenesis process. It was not clear whether the MSCs we isolated included MAPCs; nevertheless, the cell could excrete CK18, AFP and albumin, which functions were similar to that of hepatocytes. AFP and CK18 are the characteristic proteins expressed during hepatocyte development. In addition, the method we used was convenient, easy to perform, not expensive and the cell still maintained cleavage and differentiation activity after numerous generations, thus it has wide application value in future.

Many investigations[8] have revealed that bone marrow stem cell (BMSC) or purified hepatic stellate cell (HSC) can differentiate into hepatocytes. To find a suitable inducing condition has become a research hotspot lately. From the results of our study, it could be inferred that the induction of MSC by the cholestatic serum was probably through humoral signal pathway. The likely mechanism is as follows: after the choledochus was ligated, acute hepatic necrosis developed, and the liver was atrophy with a large amount of ascites in abdominal cavity; then hepatocyte growth promoters such as HGF, EGF and FGF were produced; and the cholestatic serum containing those cytokines might promote differentiation of MSC.

The concentration of cholestatic serum in our study,

50 mL/L, with coincidence to the literature[9,10] had a good effect to induce hepatocytoid cell to secrete CK18, AFP and albumin. But the constituent of cholestatic serum, being complicated and with individual variation could not be detected precisely; therefore it needs further exploration to define ingredients of the cholestatic serum, and moreover, to verify the function of synthesis and secretion, as well as metabolism and non-carcinogenesis nature of the induced hepatocytoid cell. Bone marrow stem cells represent a safe and accessible source of stem cells, and the use of MSCs provides a potential treatment approach for severe liver diseases.

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**S- Editor** Wang J **L- Editor** Zhu LH **E- Editor** Ma N

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*World J Gastroenterol* 2006 August 14; 12(30): 4870-4872
***World Journal of Gastroenterology*** ISSN 1007-9327


*RAPID COMMUNICATION*

# Efficacy of mycofenolate mofetil for steroid-resistant acute rejection after living donor liver transplantation

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## Abstract

**AIM:** To discuss the use of mycophenolate mofetil (MMF) as an immunosuppressant in steroid resistant rejection after liver transplantation.

**METHODS:** The clinical records of 260 adult patients who underwent living donor liver transplantation (LDLT) were reviewed. Tacrolimus and methylprednisolone were used for primary immunosuppression. Acute rejection was first treated with steroids. When steroid resistance occurred, the patient was treated with a combination of steroids and MMF. Anti-T-cell monoclonal antibody was administered to patients who were not responsive to steroids in combination with MMF.

**RESULTS:** A total of 90 (35%) patients developed acute rejection. The median interval time from transplantation to the first episode was 15 d. Fifty-four patients were steroid resistant. Forty-four patients were treated with MMF and the remaining 10 required anti-T-cell monoclonal antibody treatment. Progression to chronic rejection was observed in one patient. Bone marrow suppression and gastrointestinal symptoms were the most common side effects associated with MMF use. There was no significant increase in opportunistic infections.

**CONCLUSION:** Our results demonstrate that MMF is a potent and safe immunosuppressive agent for rescue therapy in patients with acute rejection after LDLT.



**Key words:** Tacrolimus; Rejection; Liver transplantation

Akamatsu N, Sugawara Y, Tamura S, Matsui Y, Kaneko J, Makuuchi M. Efficacy of mycofenolate mofetil for steroid-resistant acute rejection after living donor liver transplantation. *World J Gastroenterol* 2006; 12(30): 4870-4872

http://www.wjgnet.com/1007-9327/12/4870.asp

## INTRODUCTION

In the majority of transplant centers worldwide, the standard primary immunosuppressive regimen after liver transplantation is based on calcineurin inhibitors (CNIs) and steroids[1]. CNIs exhibit a broad spectrum of nonimmunologic side effects, including renal dysfunction, arterial hypertension, and diabetes mellitus[2]. Despite its potent immunosuppressive effect, acute cellular and chronic rejection can still occur in patients taking CNIs, even when appropriate CNI blood trough levels are maintained[1].

Mycophenolate mofetil (MMF), an enzyme in the guanine nucleotide synthetic pathway, inhibits the proliferation of both B and T lymphocytes[3]. MMF is now accepted as a promising immunosuppressant for liver transplantation. Previous reports have described its efficacy as a CNI-sparing drug to reduce CNI-related toxicity in long-term survivors[4-16]. In contrast, the role of MMF in the immediate posttransplant period is unclear[17-19]. Here, we describe our experience using MMF for patients complicated with steroid-resistant acute rejection after living donor liver transplantation (LDLT).

## MATERIALS AND METHODS

### *Patients*

A total of 260 LDLTs (140 men and 120 women; age range: 18-67 years) were performed at the University of Tokyo Hospital between January 1996 and July 2005. The median postoperative follow-up period was 28 mo (range 1-115 mo). The most common indication was virus-related liver cirrhosis ($n$ = 112) secondary to hepatitis C virus infection ($n$ = 78) or hepatitis B virus infection ($n$ = 34), followed by immune-mediated liver cirrhosis ($n$ = 74), including primary biliary cirrhosis ($n$ = 56), autoimmune hepatitis ($n$ = 9), and primary sclerosing cholangitis ($n$ = 9).

The range of pre-operative aspartate transaminase, total bilirubin, and serum creatinine levels were 19-308 IU/L, 4-400 mg/L, and 2-44 mg/L, respectively. The

**Table 1 Target trough levels of calcineurin inhibitors and steroid dosage at Tokyo University**

| | Tacrolimus (ng/mL) | Cyclosporine (ng/mL) | Methylpredonisolone (mg/kg per day) |
|---|---|---|---|
| POD 1-7 | 15-20 | 300-350 | 20-0.75 |
| POD 8-14 | 14-16 | 250-300 | 0.5-0.3 |
| POD 15-90 | 10-15 | 200-250 | 0.3-0.12 |
| POD 91-180 | 8-10 | 150-200 | 0.08-0.12 |
| POD 180- | 5-10 | 100-150 | 0.05 |

POD: Postoperative day.

**Table 2 Outcome of patients with acute rejection stratified by the rescue treatment**

| Group | *n* | CMV antigenemia *n* (%) | Systemic infection *n* (%) | Mortality *n* (%) |
|---|---|---|---|---|
| Steriod | 36 | 14 (39) | 5 (14) | 7 (19) |
| Steroid + MMF | 44 | 18 (41) | 4 (9) | 2 (5) |
| OKT3 | 10 | 7 (70) | 4 (40) | 4 (40) |
| Total | 90 | 39 (43) | 13 (14) | 13 (14) |

CMV: Cytomegalovirus; MMF: Mycophenolate mofetil.

median score for model of end-stage liver disease was 13 (range, 4-34).

### Operative and postoperative care

Our surgical technique for recipient and donor surgery is described elsewhere[20]. All patients received tacrolimus (FK, Prograf, Astellas Pharma Inc., Tokyo, Japan) and methylprednisolone as primary immunosuppressants (Table 1). When there were FK-related adverse events[21], FK was converted to cyclosporine A (CsA). The cytomegalovirus (CMV) status of the patient was monitored by pp65 antigenemia assay and CMV infection was defined by the presence of more than 5 antigen-positive cells/50 000 white blood cells. Fungal status was monitored by (1-3)-beta-D-glucan assay and antigen assays. Systemic fungal infection was defined as a positive polymerase chain reaction assay or positive culture with the existence of infectious foci. Systemic bacterial infection was defined as a positive culture from the bloodstream or infectious foci.

### Management of rejection

Acute rejection was initially suspected by biochemical evidence of deteriorating liver function. After vascular or biliary complications were excluded, liver biopsy was performed to obtain concrete pathologic evidence of rejection. The diagnosis of acute rejection was based on internationally accepted histologic criteria[22]. Our primary treatment for acute rejection was to administer high-dose methylprednisolone (20 mg/kg per day), followed by a gradual dose reduction with the CNI trough level around the upper range of our regimen. When there was no improvement in serum liver function tests, a second biopsy was obtained to confirm the diagnosis of steroid-resistant rejection. In these cases, oral MMF was initiated at the dosage of 3 g three times a d per mo, and then gradually tapered off within 2 to 6 mo. No reduction of CNIs and methylprednisolone was performed when the recipient was under MMF and after treatment with MMF. Anti-T-cell monoclonal antibody (OKT3, Ortho-Biotech Corporation, Raritan, NJ, USA) was used as a tertiary strategy for steroid-resistant refractory rejection under MMF and steroid recycle treatment.

### Statistical analysis

Patients complicated by acute rejection were divided into three groups: patients treated with one-time steroid therapy ($n = 36$), those receiving MMF administration ($n = 44$), and those eventually treated with OKT3 ($n = 10$). Inter-group comparisons were performed using the chi-square test or Fisher's exact test for categorical variables. A $P$ value of less than 0.05 was considered statistically significant.

## RESULTS

### Outcome

A total of 90 out of 260 patients developed acute rejection (35%, 90/260). The median interval time from transplantation to the primary episode of acute cellular rejection was 15 d (range 5-637 d). Fifty-four patients presented with steroid-resistant rejection and were treated with a second steroid recycle in combination with MMF. The median duration of MMF administration in these 54 patients was 74 d (range 36-182 d). Of the 54 patients who received MMF, 10 had refractory acute rejection requiring the use of OKT3. The median interval between the addition of MMF and the use of OKT3 was 5 d (range 2-8 d). Among the patients treated with OKT3, two required the additional use of basiliximab (Simulect, Novartis Pharma, Tokyo, Japan). Chronic rejection was observed in one patient (0.04%, 1/260) who eventually required re-transplantation. Graft failure due to uncontrollable acute rejection was experienced in one patient (0.04%, 1/260) who died 49 d after LDLT, despite the combined use of MMF, OKT3, and basiliximab.

### Outcome stratified by treatment

Mortality and systemic bacterial/fungal infections were significantly higher in the patients treated with OKT3 than in the other groups ($P = 0.02$ and 0.04, respectively). The incidence of positive CMV antigenemia tended to be higher in the patients treated with OKT3, although the difference was not statistically significant (Table 2).

### Side effects of MMF

MMF-associated side effects were observed in 11 patients (20%), bone marrow suppression in 9 patients (17%), and gastrointestinal symptoms in 2 patients (4%). A dose reduction of MMF and granulocyte colony stimulating factor administration was sufficient for all the patients with bone marrow suppression. Gastrointestinal symptoms disappeared spontaneously under the use of MMF. Cessation of MMF was not necessary due to adverse effects.

## DISCUSSION

The results of our study together with those of other studies[17,19] demonstrate that MMF can influence the course of steroid-resistant acute rejection. The main advantage of MMF rescue therapy is the option of continuing the therapy[19]. MMF therapy can be continued in selected patients on an outpatient basis. Rejection rescue therapy with OKT3, anti-thymocyte globulins, and anti-lymphocyte globulin, in contrast, permits only limited use for a short period of time.

Another advantage of MMF is that adverse events related to MMF are infrequent and often mild, which allows for long-term administration when required. In our series, bone marrow suppression and gastrointestinal symptoms were the most common adverse events of MMF. These episodes were easily reversed by dose reduction. MMF was not associated with a significantly increased risk of opportunistic infections. These results are compatible with previous reports[5,7,11].

LDLT theoretically offers an immunologic advantage when the donors are related to the recipients[23]. The overall incidence of acute rejection, however, is similar between LDLT and deceased donor liver transplantation. Our series demonstrated that the overall incidence of steroid-resistant acute rejection was 21%, which was unexpectedly high because LDLT recipients have been reported less likely to develop steroid-resistant or chronic rejection[24]. The 'immunologic advantage' of LDLT might be smaller than previously expected.

In conclusion, the results of our retrospective study suggest that treatment with MMF might be indicated for selected patients with acute rejection and demonstrate the high clinical value of MMF for secondary immunosuppressive therapy after LDLT.

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**S- Editor** Wang J **L- Editor** Wang XL **E- Editor** Ma N

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*World J Gastroenterol* 2006 August 14; 12(30): 4873-4874
***World Journal of Gastroenterology*** ISSN 1007-9327


*RAPID COMMUNICATION*

# Comparing mass screening techniques for gastric cancer in Japan

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Received: 2006-04-10 Accepted: 2006-05-25

Tashiro A, Sano M, Kinameri K, Fujita K, Takeuchi Y. Comparing mass screening techniques for gastric cancer in Japan. *World J Gastroenterol* 2006; 12(30): 4873-4874

http://www.wjgnet.com/1007-9327/12/4873.asp

## Abstract

**AIM:** To discuss the efficacy of endoscopic mass screening for gastric cancer.

**METHODS:** The data used in this study were the results of mass screening programs for gastric cancer in Niigata City from 2002 to 2004. The number of participants was 35089 in 2002, 34557 in 2003 and 36600 in 2004. The finding ratio referred to the final diagnosis of gastric cancer after a double check of endoscopic files and histological findings. The costs of identifying one case of gastric cancer were calculated based on the total expense for each screening program and additional close examinations.

**RESULTS:** From the analysis of individual screening program with endoscopy, individual screening program with X-ray (ISX) and mass screening program with photofluorography (MSP) in reference to the finding ratio of gastric cancer, endoscopic examination was the best for detecting early gastric cancer, the finding ratio was 0.87% in 2004, approximately 2.7 and 4.6 times higher than those of the ISX and MSP groups. In addition, this novel method was the cheapest means regarding the cost of identifying one case of gastric cancer, which was estimated to be 1608000 Japanese yen in 2004.

**CONCLUSION:** Endoscopic mass screening is a promising method and can be effectively applied if a sufficient number of skilled endoscopists become available to staff the system and if city offices support it.



**Key words:** Mass screening; Gastric cancer; Endoscopy; Cost effectiveness

## INTRODUCTION

Gastric cancer is recognized worldwide as the second leading cause of cancer mortality[1]. In Japan, non-cardia gastric cancer still remains the major cause of cancer death, although recently the mortality rate has begun to decrease [2]. With reference to this change, it has been reported that early detection of gastric cancer may contribute to decreases in the mortality rate[3,4].

Japan has carried out ongoing programs to prevent gastric cancer death by mass X-ray screening methods to detect gastric cancer in its early stages[4,5]. In recent years, endoscopy has been applied instead of X-ray as the initial mass screening method in several cities in Japan, and endoscopic mass screening has been utilized in Niigata City since 2003. The efficacy of endoscopic mass screening is discussed herein.

It is known that the finding ratio of early gastric cancer with endoscopy is higher than that with X-ray[6], making endoscopy a highly effective screening method[7]. There are, however, cost problems related to applying endoscopic mass screening[6,7]. Endoscopic screening is more expensive than direct X-ray screening (ordinary upper GI) in the Japanese medical system. Although the balance is approximately 3400 Japanese yen, because of financial problems in city offices, at the start of the present study, the costs for the two screening procedures were decided to be the same for a while.

## MATERIALS AND METHODS

### *Subjects*

The data used in this study were the results of mass screening programs for gastric cancer in Niigata City from 2002 to 2004. The number of participants was 35089 in 2002, 34557 in 2003 and 36600 in 2004. As employees have their own mass screening system of Japan, nonemployees participated in these mass screenings. All screening participants aged 40 or over could choose one of the three programs utilizing endoscopy (ISE), direct X-ray (ISX) and photofluorography (MSP: indirect X-ray

**Table 1 Transition before and after adoption of endoscopic screening program**

| | | 2002 | 2003 | 2004 |
|---|---|---|---|---|
| Participants (*n*) | ISE | - | 8118 | 11 679 |
| | ISX | 28 332 | 20 058 | 19 011 |
| | MSP | 6757 | 6381 | 5910 |
| Detection rate (%) | ISE | - | 0.81 | 0.87 |
| | ISX | 0.33 | 0.31 | 0.32 |
| | MSP | 0.25 | 0.22 | 0.19 |
| Rate of early gastric cancer (%) | ISE | - | 74.2 | 75.5 |
| | ISX | 40.8 | 56.5 | 63.9 |
| | MSP | 46.7 | 71.4 | 54.5 |
| Cost (J YEN) | ISE | - | 1 693 000 | 1 608 000 |
| | ISE$^2$ | | 2 113 000 | 1 998 000 |
| | ISX | 5 113 000 | 4 365 000 | 4 177 000 |
| | MSP | 2 712 000 | 2 792 000 | 3 290 000 |

ISE: Individual screening program with endoscopy; ISE$^2$: Cost analysis with ordinary endoscopic examination fee; ISX: Individual screening program with X-ray; MSP: Mass screening program with photofluorography; Cost: Total cost of finding one gastric cancer patient.

with a small sized film) each.

### Analysis

The finding ratio referred to the final diagnosis of gastric cancer after a double check of endoscopic files and histological findings. The costs of identifying one case of gastric cancer with each method were calculated based on the total expense for each screening program and additional close examinations.

Every citizen participates in some type of a medical insurance program, which enables all Japanese have access to medical services for next close examinations at the same costs. X-ray examination of suspected gastric cancer in ISX and MSP groups showed that the patients with suspected lesion could be referred to the endoscopists to receive endoscopy with biopsy for getting a final histogical diagnosis. We included these close examination fees in addition to the screening fee for cost analysis. In ISE group the costs of endoscopic screening and additional biopsy at the same time (if needed) were also counted.

## RESULTS

### Finding ratio

In 2004, the finding ratio in the ISE group was 0.87%, approximately 2.7 and 4.6 times higher than those of the ISX and MSP groups respectively and the rate of early gastric cancer in the ISE group was the highest among the three groups.

### Cost analysis

The cost of identifying one case of gastric cancer was estimated to be 1 608 000 Japanese yen in ISE, 4 177 000 Japanese yen in ISX, and 3 290 000 Japanese yen in MSP in 2004. In the same condition of ordinary medical fee for endoscopic examination, the cost of ISE increased to 1 998 000 Japanese yen.

These results indicated that endoscopic mass screening was the best and cheapest means for detecting and identifying gastric cancer. The results of each mass screening are shown in Table 1.

## DISCUSSION

To decrease cancer death, early detection and subsequent surveillance are necessary[8]. Although eradication of *H pylori* infection is the most effective, the incidence of gastric cancer would remain high for several decades to come[9]. Endoscopic mass screening for gastric cancer is effective in identifying cancer in its early stages[6], but endoscopic screening is more expensive than direct X-ray screening in the Japanese medical system. In this study our cost analysis showed that even if we applied ordinary examination fee for endoscopic mass screening, this novel approach was still the most superior method in cost-effectiveness for finding gastric cancer patients.

On the other hand, endoscopic mass screening is practically not appropriative for wide application due to the limited number of skilled endoscopists[6]. In Niigata City, however, we fortunately have enough endoscopists who have the title of authorized specialists as a board-certified member to examine more than 10 000 cases yearly, making it feasible to adopt an endoscopic screening system.

In mass screening systems for gastric cancer, endoscopic examination is a promising method and can be effectively applied if a sufficient number of skilled endoscopists become available to staff the system and if city offices support it.

## ACKNOWLEDGMENTS

The authors thank Dr. Kazuei Ogoshi for his assistance and advice in managing the endoscopic mass screening programs and interpretation of the results.

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**S- Editor** Wang J **L- Editor** Wang XL **E- Editor** Ma N

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*World J Gastroenterol* 2006 August 14; 12(30): 4875-4878
***World Journal of Gastroenterology*** ISSN 1007-9327


*RAPID COMMUNICATION*

# Factors predicting poor prognosis in ischemic colitis

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Received: 2005-11-03 Accepted: 2005-12-25

## Abstract

**AIM:** To determine the clinical, analytical and endoscopic factors related to ischemic colitis (IC) severity.

**METHODS:** A total of 85 patients were enrolled in a retrospective study from January 1996 to May 2004. There were 53 females and 32 males (age 74.6 ± 9.4 years, range 45-89 years). The patients were diagnosed as IC. The following variables were analyzed including age, sex, period of time from the appearance of symptoms to admission, medical history, medication, stool frequency, clinical symptoms and signs, blood tests (hemogram and basic biochemical profile), and endoscopic findings. Patients were divided in mild IC group and severe IC group (surgery and/or death). Qualitative variables were analyzed using chi-square test and parametric data were analyzed using Student's *t* test ($P < 0.05$).

**RESULTS:** The mild IC group was consisted of 69 patients (42 females and 27 males, average age 74.7 ± 12.4 years). The severe IC group was composed of 16 patients (11 females and 5 males, average age of 73.8 ± 12.4 years). One patient died because of failure of medical treatment (no surgery), 15 patients underwent surgery (6 after endoscopic diagnosis and 9 after peroperatory diagnosis). Eight of 85 patients (9.6%) died and the others were followed up as out-patients for 9.6 ± 3.5 mo. Demographic data, medical history, medication and stool frequency were similar in both groups ($P > 0.05$). Seriously ill patients had less hematochezia than slightly ill patients (37.5% *vs* 86.9%, $P = 0.000$). More tachycardia (45.4% *vs* 10.1%, $P = 0.011$) and a higher prevalence of peritonism signs (75% *vs* 5.7%, $P = 0.000$) were observed in the severe IC group while the presence and intensity of abdominal pain were similar between two groups. Two patients with severe IC had shock when admitted. Regarding analytical data, more seriously ill patients were found to have anemia and hyponatremia than the mildly ill patients (37.5% *vs* 10.1%, $P = 0.014$ and 46.6% *vs* 14.9%, $P = 0.012$, respectively). Stenosis was the only endoscopic finding that appeared more frequently in seriously ill patients than in slightly ill patients (66.6% *vs* 17.3%, $P = 0.017$).

**CONCLUSION:** The factors that can predict poor prognosis of IC are the absence of hematochezia, tachycardia and peritonism, anemia and hyponatremia and stenosis.



**Key words:** Ischemic colitis; Hematochezia; Tachycardia; Peritonism; Anemia; Hyponatremi ; Stenosis

Añón R, Boscá MM, Sanchiz V, Tosca J, Almela P, Amorós C, Benages A. Factors predicting poor prognosis in ischemic colitis. *World J Gastroenterol* 2006; 12(30): 4875-4878

http://www.wjgnet.com/1007-9327/12/4875.asp

## INTRODUCTION

Ischemic colitis (IC) is the most frequent form of vascular alterations in the digestive system. It occurs mainly in the elderly with pluripathology when there is no major vascular occlusion as a result of reduced blood flow responsible for the colon's needs, and is conditioned by many factors[1].

Clinical presentations vary from mild and limited forms not needing medical treatment to fulminant trans-mural colonic necrosis which may lead to death of patients. The variability in presentations of IC makes epidemiologic research difficult in the general population. The most frequently observed symptoms are abdominal pain and hematochezia[2].

If IC is clinically suspected, its diagnosis can be established by colonoscopy. The most frequent locations are the splenic flexure, the descending colon and the sigmoid colon, although any segment of the colon can be affected[3].

Evolution of IC in patients depends on the severity of the presentation. Most mildly affected patients are asymptomatic after medical treatment. Severely affected patients and those that have strictures need to undergo surgery and have a higher morbidity and mortality rate.

The possibility of establishing prognostic factors promptly is of great importance in deciding the best therapeutic strategy for each case. However, only a few

studies on the possible relation of etiological, pathogenic and clinical factors with the progression to colitis are available[1,3-11].

This study was to review the clinical, biological and endoscopical data of ischemic colitis patients admitted to our hospital and to discover the prognostic factors that determine the evolution of ischemic colitis.

## MATERIALS AND METHODS

### Patients

A total of 85 patients who were admitted to our hospital from January 1996 to May 2004 and diagnosed with ischemic colitis were restrospectively analyzed. The diagnosis was suspected based on the clinical and X-ray findings and confirmed by the endoscopic and histologic results or the exploratory laparotomy findings. The patients with ischemic colitis secondary to vascular surgery, thromboembolism of the mesenteric artery and/or recto-colonic tumour were excluded.

### Methods

The clinical variables including age, sex, period of time from the onset of symptoms to admission and colonoscopy, personal medical history (especially cardiovascular and metabolic history), medication taken regularly, previous stool frequency, symptoms at onset (general well being, abdominal pain, and hematochezia) were analyzed. The exploration signs on admission including hemodynamic state, especially peripheral collapse (systolic blood pressure < 90 mmHg with signs of decreased peripheral blood flow and high blood pressure (systolic blood pressure > 140 mmHg and/or diastolic blood pressure > 90 mmHg) and abdominal exploration, mainly signs of peritoneal irritation such as rigidity and rebound tenderness (Blumberg's sign) were evaluated.

The usual biological, hematological and biochemical parameters obtained on admission were also analyzed. The normal ranges of values provided by our hospital laboratory were used for the qualitative analysis of the main blood test parameters (basic hemogram and biochemical markers).

The endoscopic and laparoscopic results were described identifying the segment of the affected colon. The colon was divided into ascending colon (superior mesenteric artery) and descending colon (inferior mesenteric artery) according to the artery responsible for each segment, sigmoid and descending colon, transverse colon and ascending colon according to the location.

Patients were divided in two groups: severe ischemic colitis patient group (who needed surgery or died in the episode), and mild ischemic colitis patient group (good evolution with medical treatment). Medical treatment included fasting, symptomatic treatment, hydro-electrolytic correction and support, and use of wide spectrum antibiotics.

### Statistical analysis

Data were introduced and analyzed using SPSS version 11.0. Qualitative variables were analyzed with the chi-square test (Fisher's correction when necessary) and the parametric data were analyzed with the Student $t$ test for independent groups. Variables with $P < 0.1$ in the univariant analysis were submitted to logistic regression analysis. $P < 0.05$ was considered statistically significant.

## RESULTS

### Patients

The study included 85 patients (53 women and 32 men) with an average age of 74.6 ± 9.4 years (range 39-89 years). The main risk factor was high blood pressure which was present in 55 patients (64.7%), followed by other cardiovascular diseases (37.6%), diabetes (29.4%), chronic obstructive pulmonary disease (11.8%) and chronic renal insufficiency (1.2%). Previous cardiovascular illness was confirmed in 80% of the patients. Medications taken during the 15 d before the diagnosis of ischemic colitis were registered: antihypertensive medication (64.7%), nonsteroidal antiinflammatory drugs (NSAID) (35.3%), diuretics (25.9%), anticoagulants (3.5%) and cardiotonic drugs (2.4%). Chronic constipation was confirmed in 52 out of 71 patients (73.2%), while regular laxative intake was verified in 39 out of 66 patients (59.1%). The main symptoms that motivated medical attendance were abdominal pain (72/85 patients, 84.7%) and hematochezia (66/85 patients, 77.6%). Immediate physical exploration revealed that two patients suffered from shock at admission (systolic blood pressure < 90 mmHg), while 47.5% of the patients had high blood pressure. Abdominal exploration was compatible with peritonism (positive Blumberg's sign) in 16 cases (18.8%).

The diagnosis was established by endoscopy with biopsies (73 patients) and surgery (12 patients). A conventional abdominal ultrasound was performed in 43 patients (50.6%) and a CT-scan was done in 19 patients (22.4%) prior to endoscopy and/or surgery.

The lesions were distributed depending on their locations: descending colon in 66 patients (77.6%), splenic angle in 10 patients (11.8%), transverse colon in 4 patients (4.7%) and ascending colon in 4 patients (4.7%).

Mild ischemic colitis was found in 69 patients (81.2%) including 27 men and 42 women with an average age of 74.7 ± 8.6 years (range 45-89 years). All the patients improved after medical treatment. The severe ischemic colitis patient group was consisted of 16 patients (18.82%) including 5 men and 11 women, with an average age of 73.8 ± 12.4 years (range 39-85 years). One patient died due to failure of the medical treatment, while 15 patients underwent surgery (6 patients wo were diagnosed by colonoscopy deteriorated despite medical treatment and 9 patients were directly diagnosed during surgery). Eight patients (9.6%) died. The rest of the patients were followed up at the out-patient clinic for an average period of 9.6 ± 3.5 mo (1-72 mo).

### Severity prediction factors

The comparative analysis of mild and severe IC showed no statistically significant differences with respect to age, sex, past medical history, previous medication, stay in hospital,

**Table 1 Clinical characteristics of the patients with ischemic colitis**

| | Total *n* = 85 | Mild *n* = 69 | Severe *n* = 16 |
|---|---|---|---|
| Age (yr) | 74.6 ± 9.4 | 74.7 ± 8.6 | 73.8 ± 12.4 |
| Sex (M/F) | 32/53 | 27/42 | 5/11 |
| Hospital stay (d) | 8.5 ± 6.8 | 8.0 ± 5.5 | 10.4 ± 10.9 |
| HBP, *n* (%) | 55 (64.7) | 48 (69.6) | 7 (43.8) |
| Diabetes, *n* (%) | 25 (29.4) | 20 (29) | 5 (31.3) |
| Cardiovascular illness, *n* (%) | 32 (37.6) | 26 (37.7) | 6 (37.5) |
| CRF, *n* (%) | 1 (1.2) | 1 (1.4) | 0 (0) |
| COPD, *n* (%) | 10 (11.8) | 6 (8.7) | 4 (25) |
| Constipation, *n* (%) | 52 (61.2) | 42 (60.9) | 10 (62.5) |
| Hematochezia, *n* (%) | 66 (77.6) | 60 (87) | 6 (37.5)[b] |
| Abdominal pain, *n* (%) | 72 (84.7) | 57 (82.6) | 15 (93.8) |
| Peritonsism, *n* (%) | 16 (18.8) | 4 (5.8) | 12 (75)[b] |
| Systolic BP (mmHg) | 142.01 ± 28.3 | 145.2 ± 28.8 | 128.4 ± 22.2 |
| Diastolic BP (mmHg) | 77.7 ± 13 | 79.2 ± 13.3 | 71.2 ± 9.5 |
| Heart rate | 82.2 ± 16.2 | 80.1 ± 13.9 | 93.5 ± 22.3[a] |
| Anemia | 13 | 7 | 6[a] |
| Leukocytosis (> 108 × $10^9$/L) | 73 | 60 | 13 |
| Uremia | 26 | 23 | 3 |
| Hyponatremia | 17 | 10 | 7[a] |

[a]$P < 0.05$, [b]$P < 0.01$. Hronic renal failure, COPD: Chronic obstructive pulmonary disease.

stool frequency and location of the lesion in the intestine (Table 1).

Regarding the clinical presentation of the illness, statistically significant differences were observed between the two groups. Hematochezia was most frequently seen in the mild IC patient group compared to the severe IC patient group(86.9% *vs* 37.5%), while signs of peritonism were mostly found in the severe IC patient group compared to the mild IC patient group (75% *vs* 5.7%, $P < 0.001$). No differences were observed in the presence and intensity of abdominal pain (Table 1).

On admission, tachycardia (heart rate > 90 b/min) was found most frequently in the severe IC patient group compared to the mild IC patient group (45.4% *vs* 10.1%, $P < 0.008$). The average blood pressure (systolic and diastolic) was higher in the mild IC patient group than in the severe IC patient group (145.2 ± 28.8 mmHg and 79.2 ± 13.3 mmHg *vs* 128.4 ± 22.2 mmHg and 71.2 ± 9.5 mmHg, $P < 0.05$). However, the number of patients with high blood pressure was similar in both groups. The two patients suffering from shock at admission had severe IC (Table 1).

Biological parameter analysis showed that there were statistically significant differences between the two groups in haemoglobin (normal 120-160 g/L), platelet (normal 130 × $10^9$/L-400 × $10^9$/L) and glycemia (3.9-6.2 mmol/L) values, and similar results in the rest of the parameters (Table 1). Patients with severe IC had anemia most frequently (haemoglobin < 120 g/L) when compared to patients with mild IC (37.5% *vs* 10.1%, $P = 0.012$) and hyponatremia (serum sodium < 136 mmol/L) (46.6% of the severe *vs* 14.9% of the mild, $P = 0.012$).

No statistically significant differences were observed with respect to the endoscopic lesions (erythema, edemas, hematomas, ulcers and fibrin deposits) except for the strictures which were more frequent in the severe IC patient group (66.6% *vs* 17.3%, $P = 0.017$).

Logistic regression analysis showed that hematochezia was a weak predictor of mild IC (OR = 0.09, IC95% = 0.026-0.308, $P = 0.000$), while signs of peritonism (OR = 48.7, IC95% = 10.6-222.1, $P = 0.000$), tachycardia (OR = 7.36, IC95% = 1.71-31.5, $P = 0.007$), anemia (OR = 5.31, IC95% = 1.47-19.08, $P = 0.010$) and hyponatremia (OR = 4.98, IC95% = 1.47-16.8, $P = 0.010$) were associated with severe IC.

## DISCUSSION

Ischemic colitis is a well defined illness that is frequently found in elderly patients and is generally associated with clinical or therapeutic situations. The true incidence in the general population or in groups of patients with specific illnesses is not well known because it depends both on the ability of physicians to suspect the diagnosis and on the thoroughness of the diagnostic process. It was reported that the incidence is 6.1 to 47 in 100 000 inhabitants/year[12]. No systematic studies have established the association between ischemic colitis and cardiovascular pathology, chronic obstructive pulmonary disease, use of different medications and all circumstances responsible for a reduction in blood flow. Our patients had a high prevalence of these factors.

Observational and meta-analysis studies that point to a relationship between IC and irritable bowel syndrome and their treatment with antagonists of 5-$HT_3$ receptors have recently been published[13-16]. Whether they are different stages of the same process remains unclear. Treatment sequelae or the final consequence of an altered phenomenon that was previously considered functional has to be explained.

Another predisposing factor for IC may be constipation, probably due to the increased colonic luminal pressure which is responsible for worse blood flow in the colonic wall. All these factors, in addition to the progressive aging of the population, make us presume a future increase in its incidence. The diagnostic and therapeutic strategies need to be further studied.

Clinical presentation in ischemic colitis varies from mild forms that heal after medical treatment to severe forms with complete necrosis of the colonic wall requiring surgery and/or leading to death of patients. In general, the first symptoms of IC are hematochezia and abdominal pain as confirmed in our series.

Clinical, biological and/or endoscopic factors capable of promptly discriminating both groups of patients can be used to take the most appropriate therapeutic steps. Few studies have evaluated this aspect of IC[8-11].

Out of all the clinical parameters evaluated in our patients, that standing out as a possible predictor for the evolution of IC is only hematochezia. As a clinical presentation, it can protect the patient from severe IC, because in our study it was significantly more frequent

in the mild forms. The presence of tachycardia and peritonism on physical exploration (in our study significantly more frequent in the severe forms of IC) is possibly an expression of the hemodynamic instability and the colonic trans-mural injury, which could lead to perforation and subsequent peritonitis.

The only biological parameters are anemia and hyponatremia which were more frequent in severe forms of IC in our study.

In our series of patients, we did not find a relationship between the evolution of IC and the anatomical location of the lesions, regardless of the diagnostic method applied (endoscopy or surgery), which is different from that disclosed by other authors[9].

In this study, we found no relationship between age, sex and the subsequent evolution of IC as in other studies[8-9]. Pla *et al*[11] and Barouk *et al*[10] reported that they have established a worse prognosis in elderly patients. There are also incongruent results with regard to the relationship of the medical history of patients and the severity of IC. Medina *et al*[9] reported that there is no relationship, while others[8,10-11] reported that high blood pressure is a predisposing factor for the worse evolution of the illness.

The mortality of our patients was almost 10% and all the patients suffered from severe IC, differing greatly from that (66%) reported in the literature[17].

In conclusion, ischemic colitis is an illness that should be considered in any elderly patients with clinical and therapeutic risk factors, such as abdominal pain and/or hematochezia. The mortality rate of ischemic colitis is still high. We hold that it is necessary to carry out prospective and controlled studies in order to clearly detect the factors that predict the evolution form and find the best therapeutic approach.

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**S- Editor** Pan BR **L- Editor** Wang XL **E- Editor** Ma WH

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*World J Gastroenterol* 2006 August 14; 12(30): 4879-4883
***World Journal of Gastroenterology*** ISSN 1007-9327


*RAPID COMMUNICATION*

# Histopathological comparison of topical therapy modalities for acute radiation proctitis in an experimental rat model

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Received: 2005-07-11 Accepted: 2005-08-11

## Abstract

**AIM:** To evaluate the prevalent topical therapeutic modalities available for the treatment of acute radiation proctitis compared to formalin.

**METHODS:** A total of 120 rats were used. Four groups ($n$ = 30) were analyzed with one group for each of the following applied therapy modalities: control, mesalazine, formalin, betamethasone, and misoprostol. A single fraction of 17.5 Gy was delivered to each rat. The rats in control group rats were given saline, and the rats in the other three groups received appropriate enemas twice a day beginning on the first day after the irradiation until the day of euthanasia. On d 5, 10, and 15, ten rats from each group were euthanized and a pathologist who was unaware of treatment assignment examined the rectums using a scoring system.

**RESULTS:** The histopathologic scores for surface epithelium, glands (crypts) and lamina propria stroma of the rectums reached their maximum level on d 10. The control and formalin groups had the highest and mesalazine had the lowest, respectively on d 10. On the 15$^{th}$ d, mesalazine, betamethasone, and misoprostol had the lowest scores of betamethasone.

**CONCLUSION:** Mesalazine, betamethasone, and misoprostol are the best topical agents for radiation proctitis and formalin has an inflammatory effect and should not be used.



**Key words:** Acute radiation proctitis; Mesalazine; Formalin; Betamethasone; Misoprostol

Korkut C, Asoglu O, Aksoy M, Kapran Y, Bilge H, Kiremit-Korkut N, Parlak M. Histopathological comparison of topical therapy modalities for acute radiation proctitis in an experimental rat model. *World J Gastroenterol* 2006; 12(30): 4879-4883

http://www.wjgnet.com/1007-9327/12/4879.asp

## INTRODUCTION

Acute radiation proctitis is a major clinical complication of pelvic irradiation[1,2]. Rectum is exposed to high dose irradiation during radiotherapy of the rectal, cervical and prostate malignancies. The rate of complications, which decrease life quality, has been reported as high as 10%-15%[3,4].

The problem of radiation proctitis leads to prophylaxis studies. Sukralfat[5-9], anti-inflammatory agents[6,9], mesalazine[5] have been shown to be histopathological ineffective in clinical prophylaxis studies. In spite of the clinical comparative studies of these drugs there is no agreement on the therapy protocol[8,10-12].

There are several therapy modalities used for the protection and treatment of acute radiation proctitis and consequent chronic sequelae, including diet[13], sucralfate[14,15], antiinflamatuar agents like mesalazine[15,16], corticosteroids[5,10], formalin[17,18] and misoprostol[3,6]. However these medical approaches are not sufficiently effective and have only a limited benefit. The search for a more effective therapy should be continued.

The pathological features of irradiation begin in hours. Morphologic changes include loss of columnar shape, and nuclear polarity of enterocytes, epithelial degeneration, ulceration, nuclear pyknosis and karyorrhexis, crypt disintegration, mucosal edema, absent mitosis, crypt abscesses with prominent eosinophilic infiltrates[19].

The aim of this study was to compare the most prevalent topical therapeutic modalities used for acute radiation proctitis in a standard rat radiation proctitis model[20].

## MATERIALS AND METHODS

### Animals

Female Sprague Dawley rats weighing 230-285 g at the age of 6 wk were obtained from the Institute for Experimental Medicine, Istanbul University. All animals were acclimatized for 7 d prior to experimentation in the laboratory of the

**Table 1 Grading of histological changes on microscopic examination**

| Structure | Characteristic |
|---|---|
| Surface epithelium | Loss of cellular height/flattening of cells |
| | Cellular inflammatory infiltrate |
| Glands (crypts) | Luminal migration of epithelial nuclei |
| | Loss of goblet cells |
| | Mitotic activity |
| | Cryptitis (migration of segmental neutrophils through the crypt wall) |
| | Eosinophilic crypt abscesses |
| | Loss of glands |
| | Atrophy of glands |
| | Distortion of glands |
| Lamina propria | Inflammation |
| | Edema |
| | Congestion of blood vessels |

The abnormalities were assessed as normal (score = 0) or abnormal, ranked according to severity and arranged in quartiles.

**Table 2 Comparison of the histopathological scores of the rectal specimens with regard to the euthanasia days (mean ± SD)**

| | 5th d | 10th d | 15th d |
|---|---|---|---|
| Control | 7.40 ± 3.89 | 39.20 ± 3.58[1] | 35.40 ± 2.27 |
| Mesalazine | 5.60 ± 3.41 | 11.40 ± 3.37 | 22.40 ± 2.37 |
| Formalin | 10.0 ± 4.50 | 38.20 ± 3.61[2] | 37.00 ± 2.26[2] |
| Betamethasone | 3.20 ± 1.03 | 20.60 ± 3.41 | 18.20 ± 4.32 |
| Misoprostol | 4.40 ± 1.71 | 20.80 ± 2.70 | 23.6 ± 2.80[2] |

[1] Two specimens were excluded; [2] One specimen was excluded.

mentioned institute, which was maintained at 22 ± 2℃ and a relative humidity of 55 ± 10% in a constant 12 h dark/light circle. The rats were housed in standard wire cages and fed with standard rodent chow and UV sterilized tap water. The approval of the Ethical Review Committee of the Faculty was obtained and the experimental procedures in this study adhered to the Declaration of Helsinki for the care and use of laboratory animals.

After irradiation each animal was weighed and examined every 2 d until the end of the experiment. Five groups (30 rats in each group) were used. Groups were defined as follows. Rats in control group were anesthetized, restrained and taped by the tail and legs on an acryl plate, irradiated and given saline enemas. Rats in mesalazine group were irradiated, and given mesalazine enemas (Salofalk, Ali Raif, Istanbul, Turkey)[21]. Rats in formalin group were irradiated, and given formalin enemas (4%)[22]. Rats in betamethasone group were irradiated, and given betamethasone enemas (Betnesol,Glaxo-Wellcome, Hamburg, Germany)[13]. Rats in isoprostol group were irradiated, and given misoprostol enemas (5 μg/kg)[23]. The enemas, which were prepared in 1 mL volume at body temperature, were given twice a day beginning on the first day after irradiation until the euthanasia day. The enemas were applied with a soft feeding tube and then the anus was temporarily closed with digital compress for five minutes.

### *Irradiation*

Each rat was anesthetized using sodium pentobarbital intraperitoneally (40 mg/kg). Then 3 to 4 rats at a time were restrained and taped by the tail and legs on an acryl plate at a supine position. Lead shielding (5 half value layer) was used to cover the rat except for a 3 cm × 4 cm area of lower pelvis containing 2 cm length of rectum in the middle of the field. Irradiation was delivered with a cobalt -60 apparatus, the γ energy was 1.25 MeV, and a distance of 80 cm from the source to the surface was used. Dose rate of the irradiation was 121.49 Gy per min and 17.5 Gy in a single fraction was delivered to each rat. Ten rats from each group were selected randomly for gross and macroscopic examination and euthanized on d 5, 10, and 15. Two 5 mm segments of the rectum which was 1 cm proximal to anus were excised, fixed in 10% neutral buffered formalin solution and processed by routine techniques. Each specimen was stained with hematoxylin and eosin and examined under a light microscope by a pathologist who was unaware of treatment assignment.

The rectums were evaluated microscopically using a slightly modified scoring system reported by Hovdenak *et al*[7] by a pathologist who was blinded to the groups and the euthanasia days of specimens. A total of 13 characteristics of three mucosal structures (surface epithelium, glands, and lamina propria stroma) were used. The abnormalities of the 13 parameters were assessed as normal (score = 0) or abnormal, and ranked according to severity and arranged in quartiles: 1 = mildly abnormal; 2 = moderately abnormal; 3 = markedly abnormal: 4 = severely abnormal.

### *Statistical analysis*

Results were presented as mean ± SD. Relationship between the groups was assessed using analysis of variance. Tukey or Tamhane's test was chosen to test for equality of variances. *P* values less than 0.05 were considered statistically significant.

## RESULTS

There was no death during the study. For determination of the acute radiation injury and the response to the local therapy, 10 rats from each group were euthanized on d 5, 10 and 15 after the irradiation. The rats were apparently healthy until the day of sacrifice excluding three rats (2 in control group and 1 in formalin group), which "looked" ill and showed symptoms of diarrhea on the 10th d. The rectums of the rats were removed and examined grossly and histopathologically. Tissues from five specimens (including the three mentioned above) had severe damage and were inappropriate to be scored. They were thus excluded from the study.

The five samples excluded were two from control group on the 10th d due to the ischemic necrosis of the rectal mucosa, one from formalin group on the 10th d due to massive mucosal infarct, one from formalin group on the 15th d due to lymphocytic colitis, one from isoprostol group on the 15th d due to lymphoid hyperplasia and submucosal edema (Table 1).

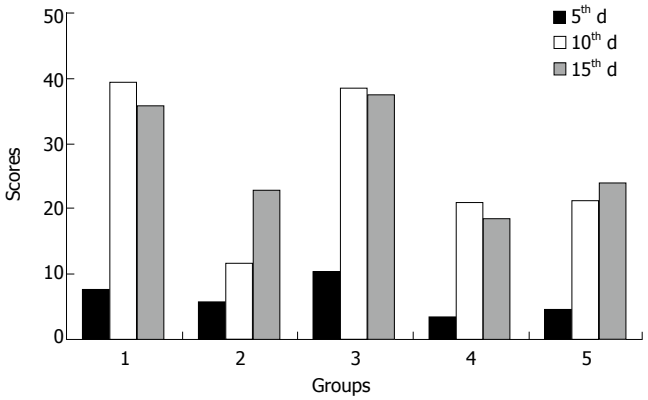


**Figure 1** Sum of the histopathologic scores regard to the euthanasia days. 1: control group; 2: mesalazine group; 3: formalin group; 4: betamethasone group; 5: misoprostol group. The differences in the mean scores of all groups, but the formalin group on the 10th and 15th d and betamethasone group on the 10th and 15th d were statistically significant.

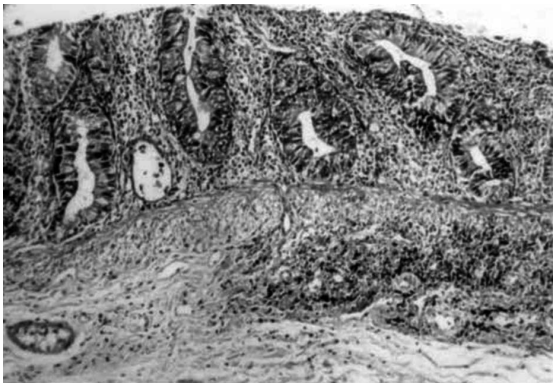

**Figure 2** Cryptitis, loss of cellular height/flattening of cells, nuclear debris in the crypt lumen and mononuclear leukocyte infiltration on lamina propria (HE X 125).

The mean histopathologic scores of the specimens are shown in Table 2.

On the 5th d the formalin group had the highest scores, followed by the control group. Mesalazine, betamethasone and misoprostol groups had the lowest scores. Table 2 gives the statistical comparison of the scores. There was no difference between control and formalin groups. The mesalazine, betamethasone and misoprostol groups had no difference between each other either. The control group had significantly higher scores than betamethasone group ($P$ = 0.039). The formalin group had higher scores than the mesalazine, betamethasone and misoprostol groups ($P$ = 0.028, 0.0001 and 0.003, respectively).

On the 10th d the control and formalin groups had the highest scores, followed by misoprostol, betamethasone and mesalazine groups (Table 2). The scores of the control and formalin groups were higher than those in the misoprostol, betamethasone and mesalazine groups ($P$ = 0.0001). There was no difference between control and formalin groups. The mesalazine group had the significantly lowest scores ($P$ = 0.0001). There was no statistically significant difference between misoprostol and betamethasone groups.

On the 15th d, the scores of formalin and control groups were the highest (Table 2). Misoprostol, mesalazine and betamethasone groups had lower scores. The formalin and control groups had significantly higher scores than the other groups ($P$ = 0.0001). There was no difference between mesalazine and misoprostol groups. The betamethasone group had the lowest scores ($P$ = 0.0001 for the control and formalin groups, and $P$ = 0.044 for the misoprostol group).

The sum of the histopathologic scores on the euthanasia days are presented on Figure 1. The inflammatory processes of the control, formalin and betamethasone groups reached a maximum score on the 10th d and decreased on the 15th d. Since nflammation of the mesalazine and misoprostol groups increased on the 10th and 15th d, the scores of the last group were higher. The mean scores of all groups, except for the formalin group and the betamethasone group on the 10th and 15th d, were significantly different.

## DISCUSSION

The aim of this study was to compare the histopathologic effects of formalin therapy with the most common agents used for radiation proctitis[4,12,18,24-26]. The consequential correlation between acute injury, which is common and usually self-limiting and chronic sequela has been demonstrated[27,28].

With a controlled radiation proctitis animal model, the four most widely used therapeutic approaches were studied in a blinded histopathologic comparison. This standard model has two handicaps: the impossibility to examine the same individual over the time and the relation of the pathologic scores to the clinical symptoms. To deal with these problems, we used groups of simultaneously sacrificed rats and a pathological scoring system obtained from a clinical study[7]. The chosen model was justified with comparison to the clinical series from the pathological point of view in regard to the duration after irradiation and the optimal dose for the human disease. The protective dose of various medications has been determined to be 17.5 Gy in single fraction[20,29].

The acute pathological findings on the rectal mucosa are reported to persist for two weeks[20,29]. An edema of the lamina propria was observed in a first couple of days, cryptitis and crypt abscesses were found on d 4 and 5, ulcers and regenerative processes could be determined on d 9 and 10, the mucosa was healed and then the chronic sequelae like fibrosis of the lamina propria arose on d 15[7].

It was reported that the pathological differences in the 10th d groups are more obvious[7]. Cryptitis (Figure 2) and crypt abscesses (Figure 3) are the predominant characteristics of acute radiation toxicity. Two specimens of the control group showed mucosal iscemic necrosis. Two specimens of the formalin group showed mucosal infarct. One specimen of the misoprostol group showed crypt hyperplasia. The mean and sum mucosal and glandular pathologic scores of the formalin group were close to those of the control group. Betamethasone and misoprostol groups had statistically significant low scores.

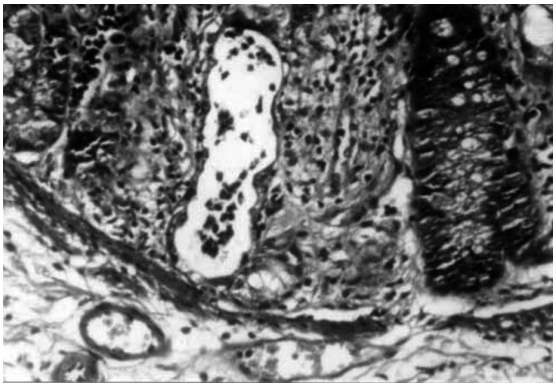

**Figure 3** Crypt abscess and nuclear debris in a crypt lumen (HE X 310).

The pathological process, determined with necrosis and edema of the mucosa and focal mucosal ulcers continued till the 15th d in euthanized animals of control and formalin groups. Betamethasone group showed the lowest pathologic scores on the 15th d.

The mucosal cytokines IL-1β, IL-2, IL-6 and IL-8 have been reported to play a significant role in the etiology of radiation-induced proctoproctitis[30]. Mesalazine and betamethasone with cytokine suppressive effects have a curative outcome on this immunology-based disease, parallel to our results. The present study showed a solely descriptive basis of the histopathology, which determines the need and extent of the clinical therapy. The immunological contribution of the disease and the effect of therapeutic approaches need to be clarified.

It should be noted that two specimens were excluded from the study, one formalin roup specimen on the 15th d due to lymphocytic colitis, one misoprostol group specimen on the 15th d due to lymphoid hyperplasia and submucosal edema. Since lymphocytic colitis, lymphoid hyperplasia and submucosal edema were encountered over the whole rectal wall of the two specimens from the same animal, the scoring of them was thus impossible. Mistol has radiation protective and tissue regenerating effects, but its mechanism of action is not fully understood[8]. We showed that it could contribute to the healing process after irradiation.

Rectal formalin therapy has serious side effects like worsening of colitis, rectal pain, anal stenosis, rectal ulcers and anal incontinence[4]. These side effects have been reported even in series with good clinical results[5,14]. These effects are in accordance with our histopathologic findings.

In conclusion, formalin should not be used in order to avoid its toxic effects on mucosa. Mesalazine and betamethasone can be used for local therapy with no major superiority between each other. Controlled randomized prospective clinical trials are required to determine the best management of this disease.

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**S- Editor** Wang J **L- Editor** Wang XL **E- Editor** Bi L

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*World J Gastroenterol* 2006 August 14; 12(30): 4884-4887
***World Journal of Gastroenterology*** ISSN 1007-9327


*RAPID COMMUNICATION*

# Seroepidemiology of hepatitis C and its risk factors in Khuzestan Province, South-West of Iran: A case-control study

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Received: 2005-08-24 Accepted: 2005-11-18

## Abstract

**AIM:** To evaluate possible risk factors for the spread of hepatitis C infection and to analyze the characteristics of the epidemiological and clinical patterns among the patients with hepatitis C infection.

**METHODS:** During a five-year period a cross-sectional study was conducted among HCV positive individuals referred to the Ahwaz JundiShapour University Hospitals (AJSUH) and Hepatitis Clinic from 1 Sept 1999 to 1 Sept 2003. The control group consisted of first time blood donors referred to the Regional Blood Transfusion organization. Enzyme-linked immunosorbent assay and recombinant immunoblot assay anti-hepatitis C virus (HCV) tests were performed for two groups. Positive serum specimens were retested using polymerase chain reaction (PCR) for HCV RNA. Risk factors were evaluated using a questionnaire. Reported risk factors among infected subjects ("HCV-positive") were compared to those of subjects never exposed ("HCV-negative") to HCV.

**RESULTS:** A total of 514 subjects were studied for HCV, of which 254 were HCV-positive and 260 HCV-negative donors comprised the control group. Mean age of the patients was 28.4 (Std 15.22) years. HCV-positive subjects were more likely to be of male gender (63% versus 37%). Transfusion 132 (52%), non-intravenous (n-iv) drug abuse and iv drug abuse 37 (14.5%), haemodialysis 25 (10%), receiving wounds at war and extramarital sexual activities (2.4%), tattooing (3.6%) were found to be independent risk factors of being HCV-positive. No apparent risk factors could be demonstrated in 29 (11.2%) of the positive cases.

**CONCLUSION:** Our data indicate that a history of transfusion and iv drug abuse and haemodialysis are important risk factors for HCV infection in our area and that more careful pretransfusion screening of blood for anti-HCV must be introduced in our blood banks. Improvements in certain lifestyle patterns, and customs in this area may be essential to prevent transmission of the infection.



**Key words:** Epidemiological patterns; Hepatitis C virus; Risk factors; South-West of Iran

Hajiani E , Hashemi J, Masjedizadeh R, Shayesteh AA, Idani E, Rajabi T. Seroepidemiology of hepatitis C and its risk factors in Khuzestan Province, South-West of Iran: A case-control study. *World J Gastroenterol* 2006; 12(30): 4884-4887

http://www.wjgnet.com/1007-9327/12/4884.asp

## INTRODUCTION

Hepatitis C virus (HCV) infection is responsible for considerable morbidity and mortality worldwide. HCV is a leading cause of liver failure and liver transplantation in adults. Identified risk factors for HCV infection include intravenous (IV) drug use, exposure to infected blood products, and intranasal drug use[1]. High-risk sexual activity [multiple sexual partners, history of sexually transmitted disease (STD)], tattooing, and skin piercing have also been suggested to be associated with increased risk for HCV[2]. In addition, mother-to-infant transmission has been demonstrated[3,4], but the possibility of other transmission routes has not been thoroughly explored. With the use of RT-PCR or bDNA techniques, HCVRNA has been detected in many systemic fluids other than blood, including peritoneal fluids, seminal and vaginal secretion, urine, and feces. These observations, however, have not been confirmed by all investigators[5]. The possibility of HCV replication in the mosquito alimentary tract has recently been demonstrated, but the epidemiological importance of this has not yet been

determined[6]. The rate of HCV infection differs in particular countries. The prevalence in developed countries amounts to 0.2%-2.2%, while in developing countries it reaches 7%. In some regions or risk group the rate of occurrence may be as high as 30%-90%. In Iran, according to the latest data, it is estimated that between 0.12-0.89 percent of the general population present anti-hepatitis C virus antibodies, which corresponds to as many as 0.5 million chronic carriers[7,8].

Risk factors associated with HCV infection may be specific to a country or region[9]. Few data are available about the risk factors associated with HCV infection in our area. Hepatitis C is a common health problem in South-West of Iran, and needs proper attention to alleviate the suffering of the people. It is essential to assess the magnitude of the problem, which will help us in understanding the dynamics of its transmission and can be utilized to guide screening procedures as well as provide insight into the control and prevention of the disease.

Herein, we report results of our cross-sectional analyses of risk factors for HCV infection in our community in Khuzestan Province, South-West of Iran conducted during a five year period.

## MATERIALS AND METHODS

During a five-year period a cross-sectional study was conducted among HCV positive individuals referred to the Ahwaz JundiShapour University Hospitals (AJSUH) and Hepatitis Clinic from 1 Sept 1999 to 1 Sept 2003. On the basis of a specially designed protocol, standard commercially available tests and physical examinations were performed. The analysis included data of medical history, physical examination and periodic evaluation clinically and serologically.

All subjects were evaluated using a face-to-face questionnaire about demographic (gender and age) and socioeconomic (education) aspects, parenteral exposure to blood or blood products, social and sexual behavior, occupational exposure, intravenous drug use, tattooing, acupuncture, surgery, previous hospitalization and parenteral administration of drugs, personal history of jaundice or hepatitis or history of these diseases in the cases' and controls' families. The control group consisted of first time blood donors referred to the Regional Blood Transfusion Organization. None of the control group subjects were HBsAg positive, HIV-positive or have any signs or symptoms of hepatitis.

Enzyme-linked immunosorbent assay (Organon/Teknica UB/HCV EIA) and recombinant immunoblot assay anti-hepatitis C virus tests were performed for two groups. Positive serum specimens were retested using polymerase chain reaction (PCR) for HCV RNA.

None of our patients showed any signs of HBV infection, or any other cause of acute or chronic liver disease, such as HAV, EBV, CMV infections, auto-immune diseases, alcohol and drug abuse, $\alpha$1-antitrypsin deficiency, Wilson's disease, or hemochromatosis. HBsAg, anti-HBc were determined by IMx analyzer (Abbott Lab., Abbott Park, IL, USA). HBsAg, anti-HBc were determined by IMx analyzer (Abbott Lab., Abbott Park, IL, USA).

**Table 1 Demographic features and symptoms and signs of liver disease of the patients enrolled into the study**

| Variable | HCV positive ($n$ = 254) | |
|---|---|---|
| | *n* | % |
| Sex | | |
| Male | 160 | 63 |
| Female | 94 | 37 |
| Age group (yr) | | |
| < 20 | 81 | 32 |
| 20-29 | 82 | 32.5 |
| 30-39 | 56 | 22 |
| 40-59 | 26 | 10 |
| > 60 | 9 | 3 .5 |
| Total | 254 | 100 |
| Symptoms | | |
| RUQ Pain | 142 | 56 |
| Malaise | 93 | 36.6 |
| Dyspepsia | 58 | 22.8 |
| Signs | | |
| Splenomegaly | 25 | 9.8 |
| Jaundice | 12 | 4.7 |
| Hepatomegaly | 8 | 3.1 |
| Elevated serum ALT level | 168 | 66 |
| HIV positive | 3 | 1.2 |

Reported risk factors among infected subjects ("HCV-positive") were compared to those of subjects never exposed ("HCV-negative") to HCV. Consent for an interview was taken from each participant, who was assured about the confidentiality of his information. Controls were briefed about the known modes of HCV transmission. The institutional Ethics Review Committee approved the study protocol.

### *Statistical analysis*

Collected data were coded, analyzed and computed, using the Statistical Package for Social Sciences (SPSS) version 10 (SPSS Inc., Chicago, IL, USA). Simple statistics such as frequency, and standard deviation were used. We conducted a multivariate logistic regression analysis to identify factors associated with HCV infection. Chi-square and Student's *t*-tests were used for comparison.

## RESULTS

A total of 514 subjects were studied for HCV, of which 254 were HCV-positive and 260 HCV-negative donors comprised the control group. Mean age of the patients was 28.4 (SD 15.22) years. Of the 254 patients, 225 (88.6%) had identifiable risks of exposure to HCV infection.

HCV-positive subjects were more likely to be of male gender (63% *vs* 37%). Transfusion 132 (52%), non-intravenous (n-iv) drug abuse and iv drug abuse 37 (14.5%), haemodialysis 25 (10%), receiving wounds at war and extramarital sexual activities 6 (2.4%), tattooing (3.6%) were found to be independent risk factors of being HCV-positive. The mean age was significantly younger in patients with transfusion (13.4 years) than the mean age of all the patients (28.4 years, $P$ = 0.004), mainly those with thalassemia and received regular blood transfusion. No

**Table 2 Risk factors possibly associated with HCV-positive in patients group**

| Variable | HCV-negative ($n$ = 260) | | HCV-positive ($n$ = 254) | |
|---|---|---|---|---|
| | $n$ | % | $n$ | % |
| Risk factor | | | | |
| Blood transfusions | 6 | 2.3 | 132 | 52 |
| Intravenous drug use | 0 | 0 | 37 | 14.5 |
| Extramarital sexual activities | 1 | 0.4 | 3 | 1.2 |
| Tattooing | 2 | 0.8 | 9 | 3.5 |
| Surgical procedures | 5 | 1.9 | 5 | 2 |
| Dental procedures | 8 | 3.1 | 12 | 4 |
| Hemodialysis | 0 | 0 | 25 | 10 |
| Receiving wounds at war | 0 | 0 | 3 | 1.2 |
| Endoscopy | 14 | 5.4 | 1 | 0.4 |
| Unknown | 224 | 86.1 | 29 | 11.2 |
| Total | 260 | 100 | 254 | 100 |

apparent risk factors could be demonstrated in 29 (11.2%) of the positive cases.

The demographic features and the background characteristics of the patient population are displayed in Table 1. Differences in the distribution of risk factors were compared between the HCV positive and negative groups, with similarities observed for educational level, economic status, and residency.

Transfusion ($P < 0.002$), intravenous drug use ($P < 0.005$) and hemodialysis ($P < 0.008$) were the only three variables with a statistically significant correlation with the presence of HCV infection on univariate analysis. There were no other statistically significant differences between the HCV positive and HCV negative control group in terms of demographic characteristics or topic areas associated with HCV infection/transmission such as extramarital sexual activities, tattooing, endoscopy, surgical and dental procedures (Table 2).

## DISCUSSION

Chronic HCV infection represents one of the major public health problems in Iran and according to the annual IBTO internal reports, it is estimated to be less than 0.2%[8,10]. Approximately 20%-30% of patients with chronic HCV infection will progress to cirrhosis[11], which can be further complicated by hepatic decompensation and development of hepatocellular carcinoma(HCC)[12,13].

In this study, 254 anti-HCV-positive patients between 1999 and 2003 were investigated. The ages of these patients, at the time of data analysis, ranged from 7 to 68 years with a mean age of 28.4 years. Our study included 160 males and 94 females, with a male:female ratio of 1:7. This male preponderance in HCV-infected patients was also reported by others[14,15].

Our study indicated blood transfusion was the leading risk factor for HCV acquisition in our patients as 52% of them were diagnosed with chronic haemolytic anemias, mainly thalassemia and received regular blood transfusion. It should be emphasized that these cases most probably, had contracted the infection before testing for HCV antibodies was performed routinely. Screening for HCV has been a routine for all blood donors since 1995 in Iran[8]. Although it is not clear whether all the HCV-positive subjects in our study population with a positive history of transfusion had had their transfusion before 1995, the date of transfusion was not determined in our study. Therefore, more careful pretransfusion screening of blood for anti-HCV must be introduced in our blood banks.

Injection drug users (IDUs) constitute the largest group of persons at high risk for acquiring HCV infection in developed countries[14,15]. Intravenous drug use was the second most frequent risk factor for HCV acquisition in our patient group (14.5%) and all of them were male, which is consistent with other reports. The male patients tended to have a history of IDU, whereas, female patients tended to have a history of transfusion[16,17]. The data on route of drug administration were based on selfreporting. One must always consider the possibility that injection drug use is underreported and that some of the HCV infection among the non-injection drug users (NIDUs) may have actually occurred through injection of drugs[18,19]. Although it is certainly possible that some of the NIDUs in this study may have injected drugs and become infected with HCV *via* injection of drugs, it does not appear that underreporting of injection drug use is a viable explanation for the results of this study.

As reported in several studies, HCV infection is a significant health problem in dialysis units in our country with a high prevalence rate (13.2%) among this population[20]. It was reported that blood transfusion and duration of dialysis treatment are important risk factors for HCV infection in patients on haemodialysis[21,22]. The more units transfused, the higher the risk for HCV infection.

Besides, we cannot exclude the possibility of nosocomial transmission of the virus over time in this group. This is supported by the fact that 8.8% of infected patients in this population had no history of blood transfusion. Patient-to-patient transmission was prospectively shown in some incidence studies in hemodialysis patients[17]. Some strategies to reduce the risk of HCV infection in patients on hemodialysis such as early screening of patients for anti-HCV, reduction of transfusion by the use of erythropoietin or screening with more sensitive methods to detect HCV; and reducing the duration of the hemodialysis period by early transplantation should be considered in this group.

No apparent risk factors could be demonstrated in 29 (11.2%) of the positive cases (called sporadic infections), which is similar to those reported previously[17]. In sporadic infections with no apparent risk factors, other routes of transmission and other factors, such as use of the same razor or tooth brush, or careless dressing of cuts and wounds or the family environment, where the infection risk increases along with exposure time should be noted[24]. Another possible explanation for this percentage of sporadic infections is failure of the questioning process in our study. We found no significant relationship between sex, previous endoscopy, receiving wounds at war, marital status with the risk of HCV infection as independent risk

factors in our cases in comparison with controls.

Although the serological, epidemiological and possible risk factors of HCV infection here obtained were related to a small population, this study was justified by the lack of information about HCV infection in Khuzestan Province, South-West of Iran. Other limitations of the study are linked to the difficulties inherent in self-reporting of behaviors such as sexual activity and drug use. It is also possible that the patients receiving blood transfusion in the study may not be representative of the population of Khuzestan Province. Genotyping was not performed in our studied cases due to limitations in our area but the genotypes of HCV were investigated in Iranian patients with histologically proven chronic hepatitis, genotype (1a) has been identified in the majority of the patients[25].

In conclusion, we provide epidemiologic features of hepatitis C and its risk factors in Khuzestan Province in South-West of Iran. These data are useful for understanding the risks of transfusion, hemodialysis, and other lifestyle patterns that predispose people to a number of HCV risk factors. These findings could be utilized to primary prevention program focused on identified risk factors, may help curtail the spread of HCV infection in this and other similar settings in developing countries. These results suggest that further study on the mode of transmission of hepatitis C should focus on patients who deny intravenous drug use or apparent risk factors. This information contributes to our understanding of the worldwide prevalence of hepatitis C. It demonstrates the importance to continue this study in order to establish routine procedures that can be applied to the screening and confirmation of the diagnosis, as well as to provide an applied methodology for the epidemiological investigation of the virologic profile of HCV-infected patients.

## ACKNOWLEDGMENTS

We thank the medical and nursing staff of Emam and Golestan hospitals for their help in the conduct of this study and the faculty of Department of Medicine of Joundishapoor University, for their encouragement and support. The authors sincerely thank the participants of the study. The authors also thank Mrs Shahnaz Shahid zadeh for her excellent assistance.

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**S- Editor** Wang J **L- Editor** Zhu LH **E- Editor** Ma N

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*World J Gastroenterol* 2006 August 14; 12(30): 4888-4891
***World Journal of Gastroenterology*** ISSN 1007-9327


*RAPID COMMUNICATION*

# Triple, standard quadruple and ampicillin-sulbactam-based quadruple therapies for *H pylori* eradication: A comparative three-armed randomized clinical trial

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Received: 2006-03-16 Accepted: 2006-04-21

## Abstract

**AIM:** To compare the effectiveness of triple, standard quadruple and ampicillin-sulbactam-based quadruple therapies for *H pylori* eradication in a comparative three-armed randomized clinical trial.

**METHODS:** A total of 360 *H pylori*-positive patients suffering from dyspepsia and aging 24-79 years with a median age of 42 years were enrolled in the study and randomly allocated into the following three groups: group A ($n$ = 120) received a standard 1-wk triple therapy (20 mg omeprazole b.i.d., 1000 mg amoxicillin b.i.d., 500 mg clarithromycin b.i.d.); group B ($n$ = 120) received a 10-d standard quadruple therapy (20 mg omeprazole b.i.d., 1000 mg amoxicillin b.i.d., 240 mg colloidal bismuth subcitrate b.i.d., and 500 mg metronidazole b.i.d.); group C ($n$ = 120) received the new protocol, i.e. 375 mg sultamicillin (225 mg ampicillin plus 150 mg sulbactam) b.i.d. (before breakfast and dinner), instead of amoxicillin in the standard quadruple therapy for the same duration. Chi-square test with the consideration of $P < 0.05$ as significant was used to compare the eradication rates by intention-to-treat and per-protocol analyses in the three groups.

**RESULTS:** The per-protocol eradication rate was 91.81% (101 patients from a total of 110) in group A, 85.84% (97 patients from a total of 113) in group B, and 92.85% (104 patients from a total of 112) in group C. The intention-to-treat eradication rate was 84.17% in group A, 80.83% in group B, and 86.67% in group C. The new protocol yielded the highest eradication rates by both per-protocol and intention-to-treat analyses followed by the standard triple and quadruple regimens, respectively. However, the differences were not statistically significant between the three groups.

**CONCLUSION:** The results of this study provide further support for the equivalence of triple and quadruple therapies in terms of effectiveness, compliance and side-effect profile when administered as first-line treatment for *H pylori* infection. Moreover, the new protocol using ampicillin-sulbactam instead of amoxicillin in the quadruple regimen is a suitable first-line alternative to be used in regions with amoxicillin-resistant *H pylori* strains.



**Key words:** Triple therapy; Quadruple therapy; Ampicillin-sulbactam; *H pylori*

Mirbagheri SA, Hasibi M, Abouzari M, Rashidi A. Triple, standard quadruple and ampicillin-sulbactam-based quadruple therapies for *H pylori* eradication: A comparative three-armed randomized clinical trial. *World J Gastroenterol* 2006; 12(30): 4888-4891

http://www.wjgnet.com/1007-9327/12/4888.asp

## INTRODUCTION

*H pylori* is responsible for the majority of peptic ulcer diseases and its eradication leads to the cure of such diseases, thereby eliminating the need for surgical treatment. Eradication of *H pylori* is indicated in the management of dyspepsia in patients under the age of 45 years without alarm symptoms (the 'test and treat' strategy) and also serves as a preventive treatment in precursor lesions of gastric cancer.

According to the Maastricht 2 guidelines, first-line eradication is triple therapy with the use of a proton-pump inhibitor b.i.d., 1 g amoxicillin b.i.d., and 500 mg clarithromycin b.i.d. In the case of penicillin allergy, 500 mg metronidazole b.i.d. is substituted for amoxicillin. When first-line *H pylori* eradication fails, a second-line treatment of quadruple therapy, with a proton-pump inhibitor b.i.d., colloidal bismuth subcitrate q.i.d., 500 mg metronidazole t.i.d., and 500 mg tetracycline q.i.d, is recommended.

Antibiotic resistance is the main cause of failure for *H pylori* eradication and bata-lactamase produced

by resistant *H pylori* strains is a possible mechanism underlying the ineffectiveness of an amoxicillin-based triple or quadruple therapy[1]. Of the 153 clinical isolates of *H pylori* found in a previous study, 71.9% are resistant to amoxicillin, 77.8% to metronidazole, and 39.2% to both[2]. The resistance rate to clarithromycin is currently 2%-30%[3]. Consequently, new treatment modalities have recently emerged to overcome antibiotic resistance[4]. However, comprehensive comparisons of the effectiveness of traditional and new treatment modalities are lacking in the literature.

Antibacterial activities of beta-lactamase inhibitors such as clavulanic acid and sulbactam have been demonstrated in a number of *in vitro* studies[5,6]. However, using clavulanic acid associated with amoxicillin has not significantly increased the *H pylori* eradication rate *in vivo*[7,8]. The aim of this study was to compare the effectiveness of the following therapeutic regimens (triple therapy, standard amoxicillin-based quadruple therapy, ampicillin-sulbactam-based quadruple therapy) in eradicating *H pylori* in a three-armed randomized clinical trial for the first time.

## MATERIALS AND METHODS

### Patients and medications

A total of 360 *H pylori*-positive patients suffering from dyspepsia and aging 24-79 years with a median age of 42 years were enrolled in the study. *H pylori* status was determined by rapid urease test at entry. After giving written informed consent, the patients were randomly allocated into three groups: group A ($n$ = 120) received a standard 1-wk triple therapy (20 mg omeprazole b.i.d., 1000 mg amoxicillin b.i.d., 500 mg clarithromycin b.i.d.); group B ($n$ = 120) received a 10-d standard quadruple therapy (20 mg omeprazole b.i.d., 1000 mg amoxicillin b.i.d., 240 mg colloidal bismuth subcitrate b.i.d., and 500 mg metronidazole b.i.d.); group C ($n$ = 120) received 375 mg sultamicillin (225 mg ampicillin plus 150 mg sulbactam, purchased from Pfizer SA, Case Postale, 8048 Zurich, Switzerland) b.i.d. (before breakfast and dinner), instead of amoxicillin in the standard quadruple therapy for the same duration. *H pylori* eradication was confirmed by $C^{14}$-urea breath test following 6 wk from the end of therapy.

All patients were contacted periodically, asked about the occurrence of possible side effects, and appropriate guidance was provided when needed. Those who were lost to follow up, or used antibiotics in the time period between the end of therapy and post-treatment urea breath test, or could not complete the treatment course because of severe side effects, were excluded in the per-protocol analysis.

### Statistical analysis

The study was a three-armed randomized clinical trial with groups A, B and C including 110, 113 and 112 patients for the final statistical analysis (per-protocol analysis). In addition, intention-to-treat analysis was also performed. Chi-square test was used to compare the eradication rates by intention-to-treat as well as per-protocol analyses in the three groups. $P < 0.05$ was considered statistically significant.

**Table 1 Demographic and clinical data of patients who completed the treatment**

| | Group A (Triple therapy) | Group B (Quadruple therapy) | Group C (New protocol) |
|---|---|---|---|
| Patients (*n*) | 110 | 113 | 112 |
| Male, *n* (%) | 57 (52 ) | 60 (53 ) | 62 (55 ) |
| Female, *n* (%) | 53 (47 ) | 53 (47 ) | 50 (45 ) |
| Age range (yr) | 28-79 (median 41) | 24-47 (median 39) | 31-68 (median 47) |
| Minor side effects, *n* (%) | 6 (5) | 5 (4) | 12 (11) |
| Overall eradication (*n*) | 101 | 97 | 104 |
| Intention-to-treat (%) | 84.17 | 80.83 | 86.67 |
| Per-protocol (%) | 91.81 | 85.84 | 92.85 |

## RESULTS

Five patients in group A, 3 in group B, and 4 in group C were lost to follow up. Four patients in group A, 2 in group B, and 4 in group C used antibiotics in the time period between the end of therapy and post-treatment urea breath test. One patient in group A and 2 in group B discontinued the regimen due to severe allergic reactions. Minor side effects were experienced by 6 patients in group A (vomiting, skin rash and abdominal pain), 5 patients in group B (vomiting, skin rash and pruritis) and 12 patients in group C (vomiting, diarrhea, headache, skin rash and abdominal pain).

Demographic and clinical details of the patients remaining in the three groups are shown in Table 1. The per-protocol eradication rate was 91.81% (101 patients from a total of 110) in group A, 85.84% (97 patients from a total of 113) in group B, and 92.85% (104 patients from a total of 112) in group C. The intention-to-treat eradication rate was 84.17% in group A, 80.83% in group B, and 86.67% in group C. The new protocol yielded the highest eradication rates by both per-protocol and intention-to-treat analyses followed by the standard triple and quadruple regimens, respectively. However, the differences were not statistically significant between the three groups. They were also not significantly different in the occurrence of minor side effects, either.

## DISCUSSION

We opted to prescribe antibiotics for ten days because 4- and 7-d regimens have been unsuccessful in Iran[9]. In this study, the eradication rates for the triple, standard quadruple and ampicillin-sulbactam-based quadruple therapies were not significantly different. Occurrence of serious side effects necessitating termination of therapy was negligible in all three groups. Minor side effects were well tolerated among all three groups and occurred infrequently with almost the same frequency. Diarrhea and headache occurred in group C only, but other side effects were experienced in all groups.

Some recent studies have compared the efficacy of triple versus quadruple therapy, and a recent meta-analysis

has assessed these studies[10]. Eradication rates were not significantly different among patients receiving triple or quadruple therapy. The duration of therapy (7 *vs* 10 d) did not significantly change the results, either. Triple therapy given for a 10-d period achieved an intention-to-treat eradication rate of 79% compared with 77% for a 7-d period. Quadruple therapy on the other hand gave an intention-to-treat eradication rate of 83% for a 10-d period and 80% for a 7-d period[10]. The eradication rates by intention-to-treat analysis among patients receiving either triple or quadruple therapy in this study were almost similar to those obtained previously[4,10,11].

A previous preliminary study by the authors using ampicillin-sulbactam instead of amoxicillin in 10-d standard quadruple therapy on 26 patients has yielded a 92% eradication rate by per-protocol analysis which was well tolerated among patients (unpublished data). The present study is the first randomized clinical trial to evaluate the efficacy of the new protocol and to compare it with standard triple and quadruple therapies in a relatively large number of patients. Although not statistically significant, the new protocol seems to be more effective than traditional protocols.

*H pylori* infection has a high prevalence rate of about 90% in Iran, which emphasizes the importance of having an effective regimen to eradicate *H pylori*[12]. The metronidazole-based standard triple therapy regimen has been unsuccessful in *H pylori* eradication, yielding an eradication rate of only about 55% compared with about 90% in other countries[13,14]. This is because metronidazole-resistant *H pylori* strains are rather common in Iran as well as in other developing countries[9,15]. The high prevalence of metronidazole-resistance in Iran could be explained by the frequent use of metronidazole to treat various infections, thereby promoting antibiotic resistance in *H pylori*.

On the other hand, 7.4% of *H pylori* isolates in Iran have been reported to be resistant against amoxicillin and higher resistance rates of up to 29% have been reported in other developing countries[15,16]. Therefore, the use of ampicillin-sulbactam instead of amoxicillin in the quadruple therapy regimen, leading to an eradication rate of 92.85% by per-protocol and 86.67% by intention-to-treat analysis in this study, may be useful against metronidazole- and amoxicillin-resistant *H pylori* strains in developing countries like Iran. Consequently, there would be no need to exclude metronidazole (because of antibiotic resistance), which is an inexpensive and widely available anti-*H pylori* agent in developing countries.

Since the present study did not show the effectiveness of the new combination on ampicillin-resistant strains, we should bear in mind that some of the resistant strains do not act through beta-lactamase but rather penicillin binding proteins (PBPs)[17]. Perhaps *in vitro* study of ampicillin-resistant strains using ampicillin-sulbactam combination can help answer whether the combination is effective against the resistant strains.

In conclusion, the results of this study provide further support for the equivalence of triple and amoxicillin-based quadruple therapies in terms of effectiveness, compliance and side-effect profile when administered as a first-line treatment for *H pylori* infection. Moreover, the new protocol using ampicillin-sulbactam instead of amoxicillin in the quadruple regimen is a suitable first-line alternative to be used in regions with amoxicillin-resistant *H pylori* strains.

## ACKNOWLEDGMENTS

The authors thank Bayat A, PhD for providing sultamicillin.

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**S- Editor** Wang J **L- Editor** Wang XL **E- Editor** Bi L

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*World J Gastroenterol* 2006 August 14; 12(30): 4892-4896
***World Journal of Gastroenterology*** ISSN 1007-9327


*RAPID COMMUNICATION*

# Prolonged intestinal mucosal acidosis is associated with multiple organ failure in human acute pancreatitis: Gastric tonometry revisited

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Received: 2005-11-21 Accepted: 2006-01-14

## Abstract

**AIM:** To evaluate whether multiple determinations of intramucosal pH (pHi) in acute pancreatitis (AP) patients could provide additional information of the disease severity during early hospitalization.

**METHODS:** Twenty-one patients suffering from acute pancreatitis were monitored by gastric tonometry in the first 72 h after hospital admission.

**RESULTS:** In the survivor group ($n$ = 15) the initially low pHi values returned to normal level (pHi $\geqslant$ 7.32) within 48 h (median pHi: d 1: 7.21; d 2: 7.32; d 3: 7.33). In contrast, pHi values in the non-survivor group $n$ = 6) were persistently either below or in the low normal range (median pHi 7.12; 7.12; 7.07 respectively), but pHi differences between the two groups reached significance only after 24 h ($P < 0.01$). Mucosal acidosis detected at any time during the monitored period was associated with the emergence of single or multiple organ dysfunction ($P < 0.01$).

**CONCLUSION:** Prolonged gastric mucosal acidosis was associated with remote organ dysfunction and failure in Acute Pancreatitis, however, correlation with the fatal outcome became significant only 24 h after admission. Due to its non-invasive nature gastric tonometry may supplement the pro-inflammatory markers to achieve a multi-faceted monitoring of the disease.



**Key words:** Acute pancreatitis; Human studies; Intramucosal pH; Mucosal barrier dysfunction; Septic complications; Bacterial translocation; Multiple organ dysfunction

Kovacs GC, Telek G, Hamar J, Furesz J, Regoly-Merei J. Prolonged intestinal mucosal acidosis is associated with multiple organ failure in human acute pancreatitis: Gastric tonometry revisited. *World J Gastroenterol* 2006; 12(30): 4892-4896

http://www.wjgnet.com/1007-9327/12/4892.asp

## INTRODUCTION

Necrotizing acute pancreatitis (AP) is currently recognized as a two-phase systemic disease, where the early phase (within the first two weeks) is characterized by sterile pancreatic necrosis and concomitant development of systemic inflammatory response syndrome (SIRS). If the patient survives the early MODS, the disease may progress to a second phase with the development of infected pancreatic necrosis and septic complications associated with multiple organ failure (MOF)[1].

Since an abundance of Gram negative bacteria[2] and other pathogens of gastrointestinal origin are commonly detected in pancreatic infections, the gut is considered to be the main source of pancreatitis related septic complications[3]. This is in accordance with experimental data suggesting that bacterial translocation through the damaged gut barrier is the most important mechanism for the contamination of pancreatic necrosis[4]. Recent evidence indicates that in case of host stress and low availability of intestinal luminal nutrition, non-pathogenic commensal intestinal bacteria can rapidly switch on virulence genes and mount a toxic offensive for their survival. Therefore, when-highly virulent traits of enteral bacteria emerge by virulence phenotype switching, gut-derived sepsis may be more likely to occur in severe AP[5].

Severe AP associated intestinal mucosal dysfunction may be the consequence of several deleterious local and systemic factors, such as disturbance of perfusion (ischemia-reperfusion phenomenon), oxidative stress during systemic inflammatory response syndrome, absence of mucosal feeding during parenteral nutrition, *etc.* This will eventually lead to metabolic failure in the gut, inducing intramucosal acidosis. Measurement of gastric intraluminal $pCO_2$ by

balloon tonometry and calculation of intramucosal pH (pHi) provides a quantitative indicator of the adequacy of intestinal milieu. Several studies have confirmed the value of pHi as a predictor of morbidity and mortality in the critically ill[6].

Gastric tonometry has been applied in human acute pancreatitis, however, the available clinical data are limited. The aim of this study was to analyze the relationship between intramucosal acidosis and AP associated complications (remote organ dysfunction and infection) with special attention to the outcome of the disease.

## MATERIALS AND METHODS

### Patients

The Ethics Committee of Semmelweis University Medical School has approved the study protocol, and written informed consent was obtained from each patient.

Gastric tonometry is generally considered to be useful in critically ill patients therefore the enrollment into this study was focused on patients hospitalized for suspected severe acute necrotizing pancreatitis . For control purposes patients with moderate and mild severity of pancreatitis were also included. The diagnosis of AP was based on laboratory findings and imaging studies (abdominal ultrasound and/or computer tomography) in association with the typical clinical picture. Exclusion criteria were as follows: pancreatitis associated with pancreatic cancer, history of recurrent AP within 3 mo, chronic, post-traumatic or post-operative pancreatitis, childhood, pregnancy, as well as administration of immune suppressive drugs (including steroids) in the previous one month. Twenty-one patients suffering from acute pancreatitis were enrolled; they were either admitted to the surgical intensive care unit (ICU) or to general surgical ward, all of them were monitored by gastric tonometry for the first three days. The end point of the study was the outcome of the disease. Based on this two groups were created in a retrospective fashion; patients with fatal outcome were enrolled in Group 1, whereas patients who survived were enrolled in Group 2.

All patients were treated according to the usual AP protocols adjusted to their current condition. Initial management was conservative. During the acute phase, the therapy consisted of adequate fluid replacement through a central venous catheter with hemodynamic monitoring, and assistance of respiratory or renal function if needed. A modified nasogastric tube was inserted to keep the stomach empty and measure intraluminal $pCO_2$. Analgesics were given as required to all patients, and they received proton pump inhibitor (PPI) (2 × 40 mg omeprazole intravenously) to prevent stress ulcers. Prophylactic antibiotics (ofloxacin/metronidazole or imipenem) were administered as soon as the presence of necrosis was evident, or CRP value was over 150 mg/L. Surgical intervention was performed when infected necrosis was diagnosed (2 cases), or if the patient's condition deteriorated despite intensive medical therapy (1 case). In one case laparoscopic cholecystectomy was performed after the patient's recovery from AP.

All patients were monitored by gastric tonometry at least twice daily during the first three days of hospitalization. Attending clinicians did not use the pHi values to guide the patients' management.

### Gastric Tonometry

Gastric tonometry was performed using a semi-automated method[7] according to the modification of the original technique described by Fiddian-Green *et al*[8]. A tonometry tube (Tonometrics™ 16F Catheter, Datex-Ohmeda Division, Instrumentarium Corp., Helsinki, Finland) was inserted into the lumen of the stomach via the nasogastric route. This catheter is a nasogastric tube with an additional smaller diameter conduit equipped with a $CO_2$ permeable silicone balloon attached to the tip. The balloon was inflated with room air and its line was connected to a bedside $CO_2$ monitor (Tonocap™ Monitor, Datex-Ohmeda). Gastric intraluminal $pCO_2$ pressure was measured after an equilibration period, and actual pHi was automatically calculated using additional data (arterial pH, arterial $pCO_2$) obtained from the patient's arterial blood samples. Since it is generally presumed that systemic metabolic acidosis confounds the interpretation of gastric pHi, all the pHi values were excluded from analysis where systemic metabolic acidosis was present. We have applied the simplified gastric pHi measurement protocol proposed by Bonham *et al*, where the daily lowest pHi value was used to characterize the mucosal condition[9]. Routine PPI administration has been employed to improve the accuracy of mucosal $pCO_2$ measurements[9], since gastric acid secretion blockade prevents the spurious elevation of luminal $pCO_2$ (resulting from the artifactual carbon-dioxide accumulation due to the reaction of bicarbonate with acid derived $H^+$-s). Mucosal acidosis was diagnosed if pHi $\leqslant$ 7.32 (the generally accepted cut off value[11]).

### Laboratory investigations

Serum amylase and lipase activities were measured by the standard spectrophotometry method (reagents from Boehringer Diagnostics, Mannheim, Germany), where the upper limit of normal values was 220 U/L and 190 U/L respectively. Acute pancreatitis was diagnosed if serum amylase level exceeded 600 U/l. CRP was determined routinely by immunoturbidimetric assay (Orion Diagnostica, Espoo, Finland); the upper limit of normal value was 10 mg/L.

### Statistical analysis

All data were presented as: median (range). Statistical analysis was performed using the Mann-Whitney *U*-test. Results were considered significant if $P$ values were $< 0.05$.

## RESULTS

Twenty-one patients with acute pancreatitis (6 women and 15 men) were involved in the study with a median age of 60 (29-77) years. The overall mortality rate was 6 of 21. There were no significant differences between the non-survivor (Group 1) and survivor (Group 2) groups in median age, gender and duration of symptoms before hospital admission. Ranson's and modified Glasgow scores showed no significant differences between the two groups, in contrast, APACHE Ⅱ scores were significantly higher in the non-survivor group from the second day after admission. Ethanol was the leading etiological factor in the first group, whereas biliary origin in the second (Table 1).

In the non-survivor group 3 of the 6 patients died

**Table 1 Clinical characteristics of the patients investigated in this study**

| | Group 1 non-survivors ($n$ = 6) | Group 2 survivors ($n$ = 15) | $P$ value |
|---|---|---|---|
| Age (yr)[1] | 69 (41-77) | 52 (29-69) | 0.22 |
| Sex ratio (M/F) | 5/1 | 10/5 | |
| Duration of symptoms before admission (h)[1] | 49.5 (12-104) | 17 (4-120) | 0.09 |
| **Etiology** | | | |
| Ethanol/gallstone/other | 3/2/1 | 5/8/2 | |
| **Disease severity** | | | |
| Predicted severity by Ranson's score[1] | 5.5 (1-8) | 3 (2-7) | 0.18 |
| Predicted severity by Glasgow criteria[1] | 2.5 (2-6) | 2 (0-6) | 0.45 |
| APACHE II at the time of admission[1] | 12 (4-15) | 6 (3-16) | 0.128 |
| APACHE II on the third hospital day[1] | 14 (10-29) | 7 (0-10) | 0.005 |
| **Outcome** | | | |
| Necrosis/No necrosis/No data[2] | 5/0/1 | 5/9/1 | |
| Remote organ dysfunction[1] | 3 (2-5) | 0 (0-2) | 0.0016 |

[1] Values are represented as median (range). APACHE: Acute Physiology and Chronic Health Evaluation. [2] No CT scan or autopsy proven data of necrosis was available.

within the first 3 d (two patients due to cardiorespiratory failure, whereas the third one due to early MOF). Three other patients deceased later (on d 9, 17, 18 respectively), all of them developed sepsis and late MOF (Table 1.) Bacteria responsible for fatal septic complications were Klebsiella, Staphylococci, Enterococci, and unidentified Gram-positive cocci.

In the survivor group local complications (peripancreatic fluid collection, necrosis) evolved in 5 of 15 patients (Table 1), and remote organ dysfunction (circulatory, pulmonary) was observed in 6 cases. Blood culture positivity was observed in 8 of 15 patients. Klebsiella, Enterobacter, Pseudomonas aeruginosa, Staphylococci and alpha hemolytic Streptococci were identified in the samples.

At the time points when the tonometry measurements were performed, serum CRP levels were significantly higher in Group 1. Other inflammatory markers, such as white blood cell counts, and the manifestations of the systemic metabolic disturbances as reflected by the daily lowest arterial pH and actual base excess values did not differ significantly between the two groups (Table 2).

Using our pHi measurement protocol there were neither failures of tonometer placement, nor tonometer related complications.

In general, the daily lowest pHi values were permanently below 7.32 in the non-survivor group (Group 1), whereas pHi was normal, or only temporarily low in the survivor group (Group 2). The pHi value differences between the two groups did not reach statistical significance on the first hospital day (Figure 1); in contrast, on the second and third days pHi values in non-survivors became significantly lower (pH 7.12 and 7.07 respectively), than those of the survivors (Figure 1).

If the relationship between the daily lowest pHi values and the APACHE II scores of the corresponding day were

**Table 2 Selected markers of inflammation, values of gastric tonometry measurements, blood test results in the patient groups**

| | Group 1 non-survivors ($n$ = 6) | Group 2 survivors ($n$ = 15) | $P$ value |
|---|---|---|---|
| Markers of inflammation | | | |
| C-reactive protein (mg/L)[1] | 408 (181-427) | 234 (95-317) | 0.048 |
| White blood cell count (G/L)[1] | 19.1 (14.5-22.6) | 15.8 (10.2-23.2) | 0.14 |
| Positive bacterial hemoculture | 3 | 8 | |
| Gastric Tonometry | | | |
| Lowest pHi within the first 72 h[1] | 7.11 (6.97-7.32) | 7.31 (6.94-7.46) | 0.0001 |
| Lowest pHi during the third day[1] | 7.07 (6.97-7.25) | 7.33 (7.27-7.46) | 0.0004 |
| Arterial blood gas results | | | |
| Arterial pH[1,2] | 7.35 (7.18-7.46) | 7.38 (7.34-7.44) | 0.18 |
| Actual base excess (mmol/L)[1,2] | -9.4 (-12.5 - -4.5) | -7 (-13.3 - -1) | 0.11 |

[1] Values are median (range). pHi: Calculated gastric intramucosal pH. [2] Daily minimum values within the first 72 h.

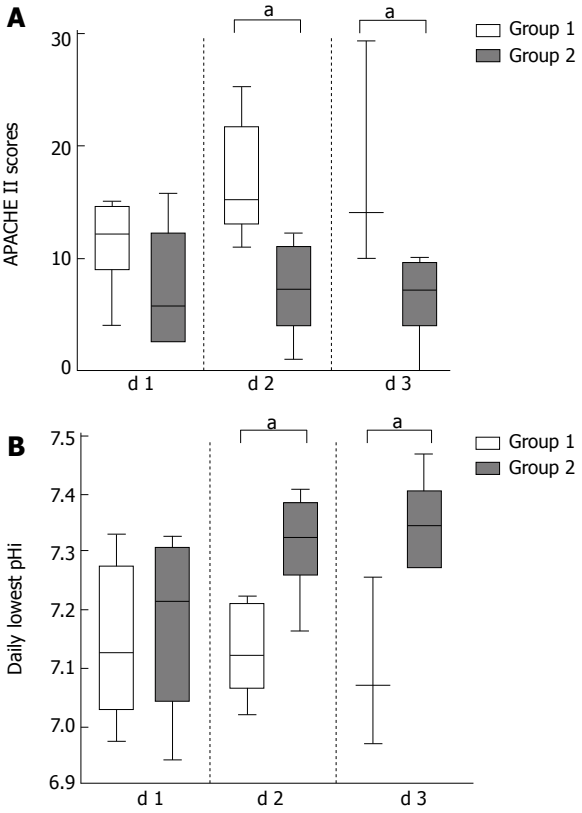


**Figure 1** The daily lowest pHi and APACHE Ⅱ values in acute pancreatitis in the survivor and non-survivor group in the first three days.

analyzed, the non-survivor group was characterized by a day-by-day decrease of pHi, which was associated with the expected rise in the APACHE Ⅱ scores. Conversely, the originally subnormal pHi values of the survivor group have returned to the normal range with a corresponding decrease in the APACHE Ⅱ scores (Figure 1).

We have found that assessment of the daily lowest pHi value was able to predict the development of non-infectious complications of acute pancreatitis. Prolonged mucosal acidosis -reflected by constantly subnormal pHi values- was associated with the development of MODS and fatal outcome. In contrast,

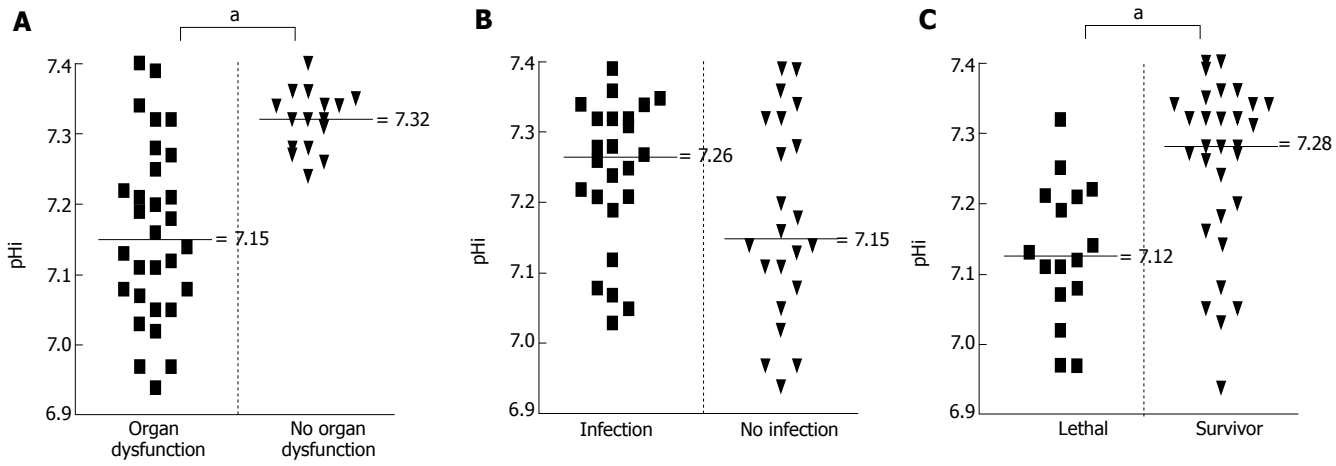


**Figure 2** Evaluation of the predictor capacity of pHi values, if single measurements are considered.

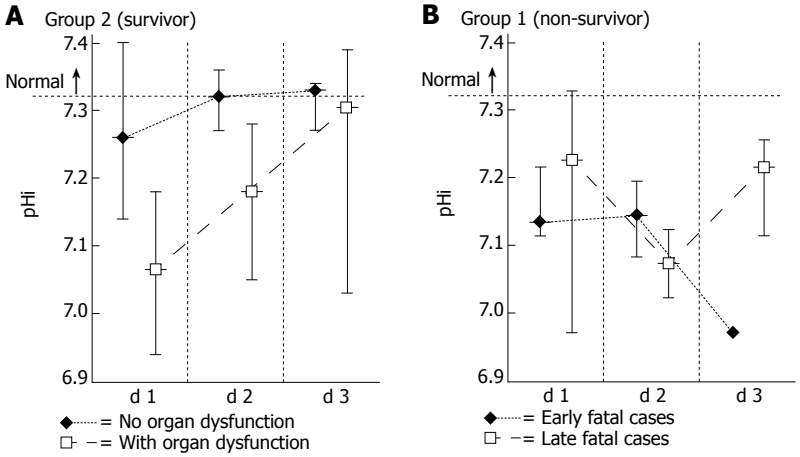


**Figure 3** Dynamics of the daily lowest pHi values in relation to the different outcomes of acute pancreatitis. In the survivor group (Group 2) pHi data were sorted to reflect the emergence or absence of MODS. In the absence of MODS pHi values stayed close to the physiological range, returning from the initial depression to normal by d 3. In patients with MODS initial pHi values were considerably lower, but a return to normal values was detectable. In the non-survivor group (Group 1) each patient developed MOF, therefore values were sorted to reflect the early or late appearance of this complication. All pHi values continued to stay at subnormal levels from the day of admission, and never returned to normal. (Values represent median and range).

in this study low pHi values were unsuitable to predict infections (Figure 2).

We have also analyzed the dynamics of the daily lowest pHi values from the viewpoint of the development of multiple organ dysfunction/failure (Figure 3). The pHi data in the survivor group were subdivided to represent the emergence or absence of MODS. By definition, MODS in this group was responding to treatment, therefore it was transient in nature. The pHi data curve in the subgroup with the absence of remote organ dysfunction characteristically started slightly below the normal value and returned to normal level by d 3. In contrast, in the subgroup of patients with remote organ dysfunction the curve started low, but similarly, a return to normal values was noted.

Since in the non-survivor group (Group 1) each patient developed MODS with deterioration into MOF (not responding to treatment), the values were sorted to reflect the early or late appearance of this complication. Although such review of the data had limited statistical power (due to the low number of cases), it could be concluded that all pHi values -irrespective of the early or late emergence of MODS/MOF- stayed at subnormal levels from d 1 and never returned to normal.

## DISCUSSION

In this study we have shown a strong correlation between persistent low gastric mucosal pHi and therapy resistant MOF associated with severe AP. This finding is in accordance with the original results of Bonham *et al*[9]. They suggested that if the lowest pHi -measured at any time during the hospitalization- was below the 7.25 cut-off value, such severe mucosal acidosis was predictive of mortality in AP.

In a study by Juvonen *et al*[12] pHi values generally did not discriminate mild AP from the severe form, but the pHi values measured at 48 h were significantly lower in the severe patient group. Our data showed that the course of gastric pHi alterations was indeed of considerable interest, since not only in mild cases, but also in patients with responsive severe disease, the originally low values returned to the normal range. This was in sharp contrast with the persistently subnormal pHi values characterizing the therapy resistant MOF cases. With respect to the early predictive capacity of gastric tonometry, however, pHi values at the time of admission did not prognosticate the outcome.

Recent advances in the comprehension of intestinal mucosal pathophysiology in AP deserve further comments. Previous studies used a somewhat simplistic framework of low-flow state and regional perfusion failure as being responsible for the development of mucosal acidosis and the related complications.

Bonham's group proposed that a mucosal ischemia-reperfusion injury was the culprit of gut barrier failure in

AP. Soong *et al*[13] emphasized the role of hyperinflammation in the pathomechanism of mucosal injury, since peak endotoxin concentrations were detected before pHi fell to its lowest level. Mucosal acidosis correlated with the consumption of endotoxin core IgM antibodies as a reflection of antecedent circulating endotoxin exposure. Hynninen *et al*[14] have found that intramucosal acidosis in severe AP was concomitant with, or rapidly followed by increases in circulating cytokines (IL-6, IL-8, and IL-10), but they could identify no correlation between endotoxemia and low pHi.

It can be suspected that further -yet unexplored- elements may influence the mucosal integrity in AP including nitric oxide dependent vasoregulatory imbalance[15], mucosal oxidative stress, up-regulation of cellular adhesion molecules, and activation of adherent polymorphonuclear leukocytes with consecutive, destructive oxygen free radical production, as well as alteration of intestinal bacterial virulence[16,17]. Consequently, acidic gastric pHi should not be considered as a simple sign of the splanchnic bed hypoperfusion, but rather as a more complex pathophysiological representation of the involvement of the gut in SIRS, evolving together with the stress related alteration in the intestinal milieu.

Although our concept of mucosal barrier failure and bacterial invasion has been enriched by new details recently, the information concerning the general well-being of the mucosa provided by gastric tonometry still seems to be of value in cases of severe AP. Cumulating evidence suggests, however, that on the other side of the mucosal barrier (i.e. in the blood and tissues) a relative immune-deficient state evolves independently from mucosal acidosis in the late course of the disease. The exaggerated SIRS is followed by compensatory anti-inflammatory response syndrome (CARS) leading to immune deactivation[18], in which the activation induced cell death (AICD) of polymorphonuclear leukocytes[19] may be an important constituent.

The additive effects of these key elements may well represent the decisive step towards sepsis, and indeed, gastric tonometry will reflect the mucosal part only. (Despite the apparent mucosal dysfunction detected in our severe AP cases, a statistically significant association between low pHi values and infectious complications could not be established in this study.)

In conclusion, intramucosal pH values showed characteristic, time dependent alterations distinguishing mild acute pancreatitis from the severe form, but detection of mucosal acidosis at admission did not improve outcome prediction. The complex sequence of pathophysiological events responsible for the development of intramucosal acidosis and septic complications is not fully elucidated in acute pancreatitis. However, future investigations should continue to benefit from gastric tonometry as a non-invasive adjunct in monitoring the intestinal mucosal function during the course of the disease.

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**S- Editor** Wang J **L- Editor** Chiarioni G **E- Editor** Bi L

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*World J Gastroenterol* 2006 August 14; 12(30): 4897-4901
***World Journal of Gastroenterology*** ISSN 1007-9327


*RAPID COMMUNICATION*

# Influence of iron on the severity of hepatic fibrosis in patients with chronic hepatitis C

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Received: 2006-03-10 Accepted: 2006-03-27

## Abstract

**AIM:** To evaluate the association among hepatic fibrosis, serum iron indices, and hepatic iron stores in patients with Chronic Hepatitis C (CHC).

**METHODS:** Thirty-two CHC patients were included in our study. The histological degree of fibrosis and inflammation activity was assessed according to the Metavir system. The serum iron indices including ferritin, iron and transferrin saturation were measured. Hepatic iron deposition was graded by Perls' stain.

**RESULTS:** The CHC patients with severe hepatic fibrosis ($n$ = 16) were significantly older than CHC patients with mild fibrosis ($n$ = 16) ($P$ = 0.024). The serum iron indices, increased serum iron store and positive hepatic iron stain were not significantly different between the two groups. In multivariate logistic regression analysis, the age at biopsy was an independent predictor of severe hepatic fibrosis (Odds ratio = 1.312; $P$ = 0.035). The positive hepatic iron stain was significantly associated with the values of alanine aminotransferase (ALT) ($P$ = 0.017), ferritin ($P$ = 0.008), serum iron ($P$ = 0.019) and transferrin saturation ($P$ = 0.003). The ferritin level showed significant correlation with the value of ALT ($r$ = 0.531; $P$ = 0.003), iron ($r$ = 0.467; $P$ = 0.011) and transferrin saturation ($r$ = 0.556; $P$ = 0.002).

**CONCLUSION:** Our findings suggest that the severity of hepatitis C virus (HCV)-related liver injury is associated with patient age at biopsy. Both serum iron indices and hepatic iron deposition show correlation with serum indices of chronic liver disease but are not related to grade and stage of liver histology.



**Key words:** Chronic hepatitis C; Hepatitis C virus; Hepatic iron; Serum iron; Hepatic fibrosis

Lin TJ, Liao LY, Lin SY, Lin CL, Chang TA. Influence of iron on the severity of hepatic fibrosis in patients with chronic hepatitis C. *World J Gastroenterol* 2006; 12(30): 4897-4901

http://www.wjgnet.com/1007-9327/12/4897.asp

## INTRODUCTION

Elevations in serum iron, ferritin and transferrin saturation are common in patients with chronic hepatitis C (CHC), as are mild increases in hepatic iron concentration. It has been reported that up to 40%-46% of patients has elevated serum iron, ferritin, or transferrin saturation level[1,2]. Although the degree of iron deposition is usually mild, histological evidence of liver iron accumulation can be observed in 10%-42.1% of patients with CHC[2-4]. Increased amounts of iron in the liver may promote the progression of liver disease by adding oxygen free radicals that increase oxidative stress[5,6]. Iron overload is responsible for liver damage through the generation of reactive oxygen species leading to lipid peroxidation and alteration of the cellular membrane[7]. Therefore, iron overload may play a role in the pathogenesis of some chronic liver diseases, especially when iron is combined with other hepatotoxic factors such as virus, free fatty acid, and alcohol[8]. In addition to the production of oxidative stress, the iron may enhance the rates of viral replication and impair the host immune system[6]. Despite these observations, the exact role of iron overload in patients with CHC remains unclear.

Factors that increase the risk of progression of hepatitis C virus (HCV)-associated hepatic fibrosis include older age at infection, male sex, alcohol abuse, and concurrent viral infection, particularly with human immunodeficiency virus or hepatitis B virus[9]. The influence of viral load and genotype on the pathogenesis of liver disease is not completely resolved. Most studies have reported that HCV RNA level has no relation to the activity of liver disease[10]. There are 6 major HCV genotypes. The most types are type 1a, 1b, 2a, 2b in Taiwan and about 65% of HCV

infections are type 1b[11]. In early studies, HCV genotype 1b was found to be associated with a more severe liver disease[12]. However, the association between genotype 1b and a more severe liver disease had not been found in studies with adjustment for the confounding factors[10,13].

Whether the degree of hepatic iron deposition in patients with CHC affects the natural history of the disease remains to be determined. The aim of this study was to assess the association among hepatic fibrosis and serum iron indices, hepatic iron stores in patients with CHC. This study was also performed to assess the other potential factors related to the severity of hepatic fibrosis in these patients, including age, gender, liver enzyme tests, viral load and genotype of HCV. We had adjusted for the other confounding factors, such as alcohol abuse, obesity, and concurrent human immunodeficiency virus or hepatitis B virus infection.

## MATERIALS AND METHODS

### CHC patients

The patients with CHC were collected at our outpatient department since October 2003. CHC was diagnosed by alteration in liver enzymes persisting for more than 6 mo associated with positive HCV antibody. Patients with potentially secondary causes of iron overload were excluded, including alcohol abuse (ethanol consumption > 20 g/d), ribavirin therapy, and multiple transfusions. The body mass index (BMI) of the patients was not over 27 kg/m$^2$. Co-infection with human immunodeficiency virus or hepatitis B virus was also excluded. Serum levels of ceruloplasmin were within normal range. Serological tests for autoimmune hepatitis (anti-nuclear antibody, anti-smooth muscle antibody) and for primary biliary cirrhosis (anti-mitochondrial antibody) were negative.

### Serological evaluation

The serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were determined by using an Olympus 5000 analyzer. The upper limit of normal for ALT is 34 IU/L. HCV antibody was detected by a commercially available enzyme-linked immunosorbent assay (AxSYM. ABBOTT Diagnostic Corporation, USA). The iron status of each patient was evaluated by biochemical tests. Serum iron (normal range, 60-160 μg/dL) was measured by the colorimetry and ferritin (normal range: 18-274 ng/mL in men and 6-283 ng/mL in women) was measured by a commercially available enzyme-linked immunosorbent assay (AxSYM. ABBOTT Diagnostic Corporation, USA). Transferrin saturation was calculated as (the serum iron divided by the TIBC) × 100%. The increased serum iron store was defined by transferrin saturation > 50% and/or ferritin > upper normal limit.

### Hepatic fibrosis stage

The hepatic specimens were obtained with the SURECUT needle by ultrasonography-guided biopsy of liver. The degree of fibrosis and inflammation activity was assessed according to the Metavir system[14]. The Metavir system scores both necroinflammatory changes on a 4-point scale of 0 to 3 and fibrosis on a 5-point scale from 0 to 4.

### Hepatic iron deposition

The histological assessment of hepatic iron stores was revealed by Perls' stain on liver biopsy specimens[15]. Hepatic iron deposition was graded on a scale from 0 to 4. Perls' stain is also called Prussian Blue reaction. It is used to demonstrate ferric iron and ferritin. This is not a true staining technique rather, it is a histochemical reaction. The protein is split off by the hydrochloric acid, allowing the potassium ferrocyanide to combine with the ferric iron. This forms the ferric ferrocyanide or Prussian Blue.

### Viral load of HCV

The viral load of HCV was checked according to the Cobas Amplicor HCV Monitor Test, version 2.0, Roche Diagnostics. It is based on five major processes: specimen preparation; reverse transcription of the target RNA to generate complementary DNA (cDNA); polymerase chain reaction (PCR) amplification of target cDNA using HCV specific complimentary primers; hybridization of the amplified products to oligonucleotide probes specific to the targets; and detection of the probe-bound amplified products by colorimetric determination.

### Viral genotype of HCV

The most viral genotypes of HCV are type 1a, 1b, 2a, 2b in Taiwan. We used the method of type-specific PCR to analyze the viral genotype of HCV. Based on variation in nucleotide sequence within restricted regions in the putative C (core) region of HCV, four groups of HCV had been illustrated[16]. They were types 1a, 1b, 2a and 2b. The method depended on the amplification of a C gene sequence by PCR using a universal primer (sense) and a mixture of four type-specific primers (antisense). HCV types were determined by the size of the products specific to each of them. The primers of first round PCR were 5'-CGAAAGGCCTTGTGGTACTG-3' and 5'-ATATACCCCATGAGGTCGGC-3'. The primers of second round PCR were sense primer 104: 5'-AGGAAGACTTCCGAGCGGTC-3' and four antisense primers. They were antisense primer 132: 5'-TGCCTTGGGGATAGGCTGAC-3', antisense primer 133: 5'-GAGCCATCCTGCCCACCCCA-3', antisense primer 134: 5'-CCAAGAGGGACGGGAACCTC-3' and antisense primer 135: 5'-ACCCTCGTTTCCGTACAGAG-3'.

### Statistical analysis

Data were summarized as mean ± SD. Data were compared between groups on the basis of hepatic fibrosis stage. Categorical variables were compared with the chi-square test or Fisher's exact test as required. Continuous variables were compared between groups by using the unpaired *t*-test. The Mann-Whitney test was used when it was appropriate. Independent factors related to hepatic fibrosis severity were assessed by using multivariate logistic regression analysis. Correlations among selected variables were assessed by the Spearman correlation coefficient. The $P < 0.05$ was statistically significant.

**Table 1 Demographic and laboratory data of 32 patients with CHC**

| Variable | Total population ($n$ = 32) | Severe fibrosis ($n$ = 16) | Mild fibrosis ($n$ = 16) | $P$ |
|---|---|---|---|---|
| Age (yr) | 56.47 ± 10.92 | 60.75 ± 6.50 | 52.19 ± 12.85 | 0.024 |
| Sex (male:female) | 15 : 17 | 8 : 8 | 7 : 9 | 0.723 |
| AST (IU/L) | 105.47 ± 56.18 | 119.31 ± 55.75 | 91.63 ± 54.82 | 0.167 |
| ALT (IU/L) | 163.03 ± 102.99 | 167.81 ± 102.15 | 158.25 ± 106.95 | 0.798 |
| Iron (μg/dL) | 155.07 ± 43.27 | 149.50 ± 33.00 | 160.27 ± 51.71 | 0.513 |
| TIBC (μg/dL) | 356.97 ± 48.38 | 351.50 ± 41.23 | 362.07 ± 55.18 | 0.566 |
| Transferrin saturation (%) | 43.63 ± 11.69 | 42.51 ± 7.85 | 44.68 ± 14.62 | 0.621 |
| Ferritin (ng/mL) | 291.19 ± 213.72 | 329.41 ± 222.08 | 255.53 ± 206.72 | 0.362 |
| Increased serum Iron store | 14 (43.75%) | 8 (50.00%) | 6 (37.50%) | 0.476 |
| Positive hepatic Iron stain | 4 (12.50%) | 1 (6.25%) | 3 (18.75%) | 0.600 |
| Viral genotype (1:2) | 22 : 6 | 12 : 2 | 10 : 4 | 0.648 |
| Viral load ($\times 10^6$ copies/mL) | 4.94 ± 6.26 | 6.39 ± 8.16 | 3.48 ± 3.22 | 0.231 |

CHC: Chronic hepatitis C; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; TIBC: Total iron binding capacity. Data are expressed as mean ± SD or patients number (percentage). Transferrin saturation was calculated as serum iron divided by TIBC × 100%. The increased serum iron store was defined by transferrin saturation > 50% and/or ferritin > upper normal limit.

## RESULTS

Thirty-two patients fulfilling inclusion criteria were studied. The demographic and laboratory data of the patients are summarized in Table 1. The mean age of the 32 patients was 56.47 ± 10.92 year-old. Fourteen patients (43.75%) had increased serum iron stores and only four patients (12.5%) had positive hepatic iron stain. In the four patients, three patients were grade one and one patient was grade two on Perls' stain. Of 32 patients, 16 patients showed severe hepatic fibrosis (stages 3 or 4) and 16 patients had mild fibrosis (stages 0, 1 or 2) on histology.

The CHC patients with severe hepatic fibrosis were significantly older than the CHC patients with mild fibrosis (60.75 ± 6.50 *vs* 52.19 ± 12.85 year-old; $P$ = 0.024). The other variables showed in Table 1, including gender, liver enzyme tests, serum iron indices, increased serum iron store, positive hepatic iron stain, viral load and genotype of HCV, were not significantly different between patients with severe and mild hepatic fibrosis. In multivariate logistic regression analysis, the age at biopsy was still an independent predictor of severe hepatic fibrosis (Odds ratio = 1.312; $P$ = 0.035) (Table 2).

We stratified our data according to patient sex because women may have lower serum iron markers than men. All the serum iron indices and hepatic iron stain were not associated with severe hepatic fibrosis in men and women, respectively. Univariate analysis across grades of histological inflammation activity also did not show a significant association between inflammation activity and any of the serum iron indices or the presence of hepatic tissue iron, age, gender, liver enzyme tests, viral load and genotype of HCV (data not shown).

The positive hepatic iron stain was significantly associated with the values of ALT ($P$ = 0.017) and all the three serum iron indices, including ferritin ($P$ = 0.008), iron ($P$ = 0.019) and transferrin saturation ($P$ = 0.003) (Table 3). The ferritin level had significant correlation with the value of ALT, iron and transferrin saturation in Spearman correlation test ($P$ = 0.003, 0.011 and 0.002, respectively). Nonetheless, no significant correlation was found between ferritin and grade of inflammation activity or stage of hepatic fibrosis severity (Table 4).

**Table 2 Multivariate logistic regression analysis of independent predictors of severe hepatic fibrosis**

| Variable | Odds ratio | 95% CI | $P$ |
|---|---|---|---|
| Age | 1.312 | 1.020-1.688 | 0.035 |
| Male gender | 14.138 | 0.835-239.266 | 0.066 |
| Increased serum iron store | 0.834 | 0.081-8.595 | 0.879 |
| Positive hepatic iron stain | 1.584 | 0.067-37.349 | 0.775 |
| Viral load | 1.412 | 0.923-2.161 | 0.112 |

CI: Confidence interval; ALT: Alanine aminotransferase. The increased serum iron store was defined by transferrin saturation > 50% and/or ferritin > upper normal limit. The ALT and viral genotype were eliminated in multivariate backward logistic regression analysis. We forcibly add the two factors increased serum iron store and positive hepatic iron stain into the analysis.

**Table 3 Univariate analysis of demographic and laboratory data in relation to hepatic iron stain**

| Variable | Positive hepatic iron stain ($n$ = 4) | Negative hepatic iron stain ($n$ = 28) | $P$ |
|---|---|---|---|
| Age (yr) | 52.50 ± 9.04 | 57.04 ± 11.19 | 0.446 |
| Sex (male:female) | 3 : 1 | 12 : 16 | 0.319 |
| AST (IU/L) | 141.50 ± 99.94 | 100.32 ± 47.89 | 0.473 |
| ALT (IU/L) | 275.00 ± 172.18 | 147.04 ± 82.23 | 0.017 |
| Iron (μg/dL) | 210.50 ± 45.11 | 146.20 ± 36.55 | 0.019 |
| Transferrin saturation (%) | 62.34 ± 10.71 | 40.64 ± 8.80 | 0.003 |
| Ferritin (ng/mL) | 591.23 ± 119.70 | 243.19 ± 184.65 | 0.008 |

Data are expressed as mean ± SD. AST: Aspartate aminotransferase; ALT: Alanine aminotransferase. Transferrin saturation was calculated as (the serum iron divided by the TIBC) × 100%.

**Table 4 Correlations with ferritin levels among laboratory data, hepatic inflammation and fibrosis scores**

| Variable | ALT | Iron | Transferrin saturation | Inflammation score | Fibrosis score |
|---|---|---|---|---|---|
| Spearman coeff | 0.531 | 0.467 | 0.556 | -0.085 | 0.317 |
| $P$ | 0.003 | 0.011 | 0.002 | 0.659 | 0.094 |

ALT: Alanine aminotransferase. Transferrin saturation was calculated as (the serum iron divided by the TIBC) × 100%.

## DISCUSSION

It has been recognized for more than 30 years that iron stores may be increased in alcoholic liver disease[17]. In nonalcoholic steatohepatitis, 58% of patients has elevated serum iron indices and in some cases increased hepatic iron stores[18]. In order to prevent the effect of

the potential confounding factors in hepatic iron stores, we carefully excluded patients that had alcohol abuse (ethanol consumption > 20 g/d), BMI over 27 kg/m$^2$, previous ribavirin therapy and multiple transfusions. The lower rate of positive hepatic iron stain (12.5%) may partly be due to the stringent selection criteria used in our study. In our previous study, we showed that the HFE mutations associated with hereditary hemochromatosis were infrequent in Taiwan and they may not contribute to iron accumulation in CHC patients even when serum iron overload was observed in more than one third of these patients[19]. Therefore, we didn't exclude the few CHC patients with HFE mutations in our study.

Although the iron-related oxidative stress may play a role in the pathogenesis of CHC, the association between serum iron markers, hepatic iron stores, and hepatic fibrosis stage remains controversial. Previous studies had evaluated the potential impact of hepatic iron store on CHC but they had produced discordant results. Three studies had found that hepatic iron tissue deposition was associated with severe hepatic fibrosis in patients with CHC[20-22]. Despite the association, they did not found a correlation between the amount of hepatic iron store and the fibrosis score. The absence of dosing effect suggests that there is a cut-off point at which all patients are more likely to have severe fibrosis, and all patients with values above this level have an equal risk regardless of the quantity of tissue iron concentration[20]. In other words, there is a threshold effect, and once present, increasing hepatic iron does not correlate with increasing fibrosis[22]. The other studies had proposed the discordant conclusions. No association was observed between the presence of hepatic iron deposition and fibrosis score in these reports[23-26]. In our study, significant iron that was detectable histologically was also unrelated to the severity of hepatic fibrosis. It is well established that a heavy iron overload per se can cause hepatic fibrosis, as observed in patients with hereditary hemochromatosis. In a semi-quantitative evaluation of hepatic iron in patients with CHC, most had minimal or mild deposits[25]. Our study had the similar results. In the four patients with positive hepatic iron stain, three patients were grade one and one patient was grade two on Perls' stain. This may be the reason why the hepatic iron stain was not associated with severe hepatic fibrosis in our study. Mild degree of hepatic iron deposition may not reach the threshold at which iron will enforce hepatic injury.

In the present study we did not find any association between serum iron indices or hepatic iron stain and degree of hepatic fibrosis or inflammation activity in patients with CHC. However, our study had showed that hepatic iron stain was associated with altered ALT values and serum iron indices. The ferritin levels also showed correlation with ALT values and the other two serum iron indices. That is, the biochemical injury of liver can be predicted by tissue or serum iron contents but the histological damage can't. This is consistent with the finding that the decline in serum AST and ALT values after phlebotomy is not associated with a change in histological activity of inflammation or fibrosis[27]. The mechanism by which iron accumulates in some patients with CHC is unclear. Whether this iron accumulation is cause or result of liver injury is unknown. Previous studies had reported a positive correlation between serum ferritin concentration and ALT level in patients with CHC[1,28]. Since serum iron index correlated significantly with the value of ALT, it was likely that the excess iron could be related to its release from destroyed hepatocytes as a result of liver injury associated with HCV. This suggested that iron parameters in patients with CHC acted either as markers of the chronic inflammatory state or cytolytic liver activity but did not directly reflect the progression of hepatic fibrosis. Furthermore, the tissue iron contents did associate with the all serum iron indices in our study. In other words, ferritin, iron and tranferrin saturation were all excellent predictors for presence of hepatic iron in patients with CHC.

Our study found that older age at biopsy was associated with severe hepatic fibrosis in patients with CHC. This suggested that hepatitis C infection may somehow become more fibrogenic with advancing host age. This is in accordance with previous studies showing that severity of HCV-related liver injury can be predicted by patient age[23,24,29]. The mechanism underlying this association is unknown. The possible explanations might include immune factors, increased fibrogenesis, or decreased fibrolysis[9]. The ability of hepatocytes to regenerate or the state of activated hepatic lipocytes alters with age and thus gives rise to increased fibrosis[29]. Nonetheless, these speculations are unproven yet. Our data allow a conclusion that CHC will place an increasing burden on health care services in the next decades as the population with CHC ages.

Our study do have a potential limitation. In two large-scale studies, age at onset of infection had been identified as predictive factor of progression in CHC[13,30]. Since the time of onset of infection derived from clinical history may not be reliable, we had omitted this variant in our study.

In conclusion, the severity of HCV-related liver injury is associated with patient age at biopsy. Significant iron deposition in the liver is uncommon in CHC patients. Both serum iron indices and hepatic iron deposition show correlation with serum indices of chronic liver disease but are not related to grade and stage of liver histology. The viral load and genotype of HCV are also not associated with hepatic fibrosis severity and inflammation activity. Our study conclusions suggest that patients with CHC should be treated as early as possible. Our findings do not support the role for iron depletion therapy by phlebotomy in patients with CHC, including those with elevated serum iron indices or positive hepatic iron stain.

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**S- Editor** Wang J **L- Editor** Chiarioni G **E- Editor** Bi L

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*World J Gastroenterol* 2006 August 14; 12(30): 4902-4905
***World Journal of Gastroenterology*** ISSN 1007-9327


*RAPID COMMUNICATION*

# Dynamical changing patterns of histological structure and ultrastructure of liver graft undergoing warm ischemia injury from non-heart-beating donor in rats

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Received: 2006-03-18 Accepted: 2006-04-21

## Abstract

**AIM:** To investigate the histological and ultra-structural characteristics of liver graft during different of warm ischemia time (WIT) in rats and to predict the maximum limitation of liver graft to warm ischemia.

**METHODS:** The rats were randomized into 7 groups undergoing warm ischemia injury for 0, 10, 15, 20, 30, 45 and 60 min, respectively. All specimens having undergone warm ischemia injury were investigated dynamically by light and electron microscopy, and histochemistry staining. After orthotopic liver transplantation (OLT), the recovery of morphology of liver grafts after 6, 24 and 48 h was observed.

**RESULTS:** The donor liver from non-heart-beating donors (NHBD) underwent ischemia injury both in the warm ischemia period and in the reperfusion period. Morphological changes were positively related to warm ischemia injury in a time-dependent manner during the reperfusion period. The results demonstrated that different degrees of histocyte degeneration were observed when WIT was within 30 min, and became more severe with the prolongation of WIT, no obvious hepatocyte necrosis was noted in any specimen. In the group undergoing warm ischemia injury for 45 min, small focal necrosis occurred in the central area of hepatic lobule first. In the group undergoing warm ischemia injury for 60 min, patchy or diffused necrosis was observed and the area was gradually extended, while hepatic sinusoid endothelial cells were obviously swollen. Hepatic sinusoid was obstructed and microcirculation was in disorder.

**CONCLUSION:** The rat liver graft undergoing warm ischemia injury is in the reversible stage when the WIT is within 30 min. The 45 min WIT may be a critical point of rat liver graft to endure warm ischemia injury. When the WIT is over 60 min, the damage is irreversible.



**Key words:** Liver transplantation; Warm ischemia injury; Morphological observation

Ma Y, Wang GD, Wu LW, Hu RD. Dynamical changing patterns of histological structure and ultrastructure of liver graft undergoing warm ischemia injury from non-heart-beating donor in rats. *World J Gastroenterol* 2006; 12(30): 4902-4905

http://www.wjgnet.com/1007-9327/12/4902.asp

## INTRODUCTION

In the past 40 years, liver transplantation has achieved a great success and has become the most effective method to treat the end-stage hepatic diseases. Liver transplantation is developing rapidly as a result of perfect perioperative care and widespread applications of potent immunosuppressants. However, there is an obvious problem of donor organ shortage, especially in countries where the "brain-death" cases have not been legitimated. At present, a high percentage of liver grafts comes from non-heart-beating donor (NHBD) in these countries. Under these circumstances, liver grafts unavoidably encounter a period of warm ischemia injury and undergo further injuries in preservation and reperfusion process[1-3]. Poor quality of liver grafts is considered an important risk factor greatly reducing the liver transplantation effectiveness[4-6]. Clinical practice suggests that the warm ischemia time (WIT) should not be longer than 5 min[7], and 10 min of WIT may be the upper limit. According to this, many donor livers are useless, thus aggravating the problem of donor liver shortage. This study was to observe the changing patterns of histological structure and ultrastructure of liver graft undergoing warm ischemia injury.

## MATERIALS AND METHODS

### *Animals and grouping*

Two hundred and ten healthy male adult Sprague-Dawley (SD) rats weighing 250-300g (Experimental Animal Center at Sun Yat-Sen University) were used in the study. The mean weight of recipient rats was slightly heavier than that of donor rats. According to WIT, 210 SD rats were randomly divided into seven groups. The duration of WIT was 0, 10, 15, 20, 30, 45 and 60 min respectively. Forty-two SD rats (6 each group) were used for the observation of warm ischemia injury. The other 168 were divided into 7 subgroups. Orthotopic liver transplantation (OLT) was performed in each group according to the predetermined WIT, 12 as donors and 12 as recipients. Histological, histochemical and ultrastructural changes were observed 6, 24 and 48 h respectively after reperfusion. Specimens taken from the right hepatic lobe of rats were divided into 4 types, one for routine olefin sections after fixation in formalin solution, one for glycogen staining after fixation in Gendre solution, one for enzyme histochemical staining after quick freezing in liquid nitrogen and one for ultrathin sections.

### *Establishment of animal model*

Warm ischemia injury was induced by clamping the basilar part of the heart and blocking the thoracic aorta of the donor animals after the donor rat received 0.2 mL heparin sodium solution (1250 U heparin sodium) *via* dorsum of penis vein to establish the non-heart-beating donor model. Then the liver graft was dissected. The liver was then perfused *in situ* through the abdominal aorta with 20 mL chilled lactic acid Ringer's solution (50 U/mL heparin sodium) and stored in a bath of cold lactate Ringer's solution before transplantation. Immediately prior to the portal vein clamping, orthotopic liver transplantation was performed as previously described[8] with minor modifications[9]. The cold ischemia time (CIT) was 50 ± 3.5 min and the anhepatic period was 20 ± 2.5 min.

### *Observation indexes and methods*

Specimens were fixed in formalin solution, routine 4-6 μm paraffin sections were stained with HE for light microscopy.

Specimens were disposed by the typical ultra-histology methods, and the sections were observed under transmission electron microscope and scanning electron microscope.

Hepatic glycogen was stained by PAS reaction after the fresh specimens were fixed in Gendre's solution for 6 h. Tetrazolium blue, PPDA, magnesium activation were respectively adopted to observe the activities of SDH, CO and ATPase on 5-6 μm thick cryo-sections.

## RESULTS

### *Observation under light microscope*

Histological structure changed slightly when WIT was shorter than 30 min. Cytoplasm loosening, cell edema, focal vacuole degeneration were noted when WIT was over 30 min, especially in the lobule center area. Leukocyte infiltration was noted in the portal area and acidophilus was obvious in some hepatocytes. The above pathologic changes aggravated when WIT was prolonged to 60 min. Cell degeneration was diffuse or extended to a focal area, even lipid degeneration could be seen. The degree of degeneration was dependent on the duration of WIT, but necrosis could hardly be observed under light microscope. After 6 and 24 h reperfusion, injury to liver graft became severer and hepatocytes presented with obvious edema and some ballooning degeneration in the group undergoing warm ischemia injury for 30 min (Figures 1A and B). Focal like necrosis could be noted in the lobule center area in the group undergoing warm ischemia injury for 45 min (Figure 1C), the change aggravated when WIT was prolonged. Forty-eight hours after reperfusion, hepatic injury resumed gradually in the group undergoing warm ischemia injury for < 45 min. Hepatocytes presented with plaque or diffused necrosis and the pathologic change was irreversible in the group undergoing warm ischemia injury for 50 min (Figure 1D).

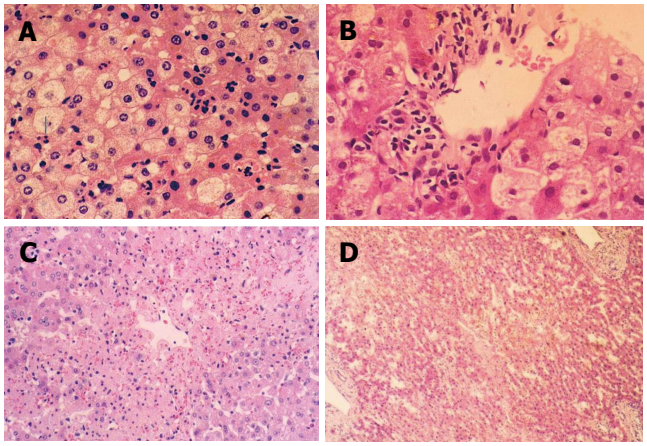


**Figure 1** Cytoplasm loosening, cell edema, focal vacuole degeneration after reperfusion (10 × 10) in the group undergoing warm ischemia injury for < 30 min (**A**); obvious cell edema, ballooning-like degeneration after reperfusion (40 × 10) in the group undergoing warm ischemia injury for 30 min (**B**); focal necrosis around central lobule area after reperfusion (10 × 10) in the group undergoing warm ischemia injury for 45 min (**C**); plaque-like area necrosis after reperfusion (40 × 10) in the group undergoing warm ischemia injury for 60 min (**D**).

### *Observation under electron microscope*

The structure of mitochondria and endoresticule was normal when WIT was shorter than 15 min (Figure 2A). Mitochondria became swollen, density of basal plasma was reduced, endoresticule was enlarged and glycogen particles were reduced in the group undergoing warm ischemia injury for 30 min. Mitochondria crista became fuzzy and pale in the group undergoing warm ischemia injury for 45 min. While fuzzy or ruptured mitochondria crista, vacuole degeneration and broken endoresticule were noted in the group undergoing warm ischemia injury for 60 min. Six hours after reperfusion, damage to liver graft became severer and 24 h after reperfusion, mitochondria became swollen and basal density reduction was aggravated, but glycogen particles increased in the group undergoing warm ischemia injury for < 30 min (Figure 2B). Mitochondria became swollen, vacuole was

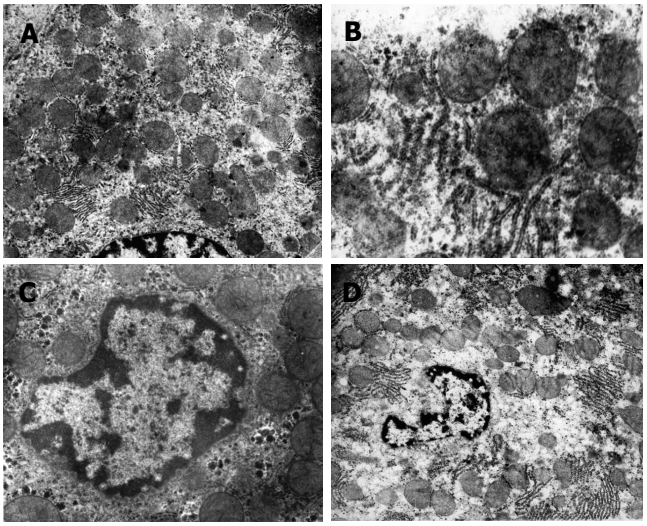


**Figure 2** Swollen mitochondria, ruptured crista, rough endoplasmic reticulum degranulation (× 15000) in the group undergoing warm ischemia injury for 30 min (**A**); phanero-swollen mitochondria and nuclear chromosome margination, and karyopyknosis after reperfusion (× 10000) in the group undergoing warm ischemia injury for 45 min (**B** and **C**); significant swollen mitochondria, crista extinction, nuclear membrane rupture, karyolysis and karyorrhexis after reperfusion (× 5000) in the group undergoing warm ischemia injury for 60 min (**D**).

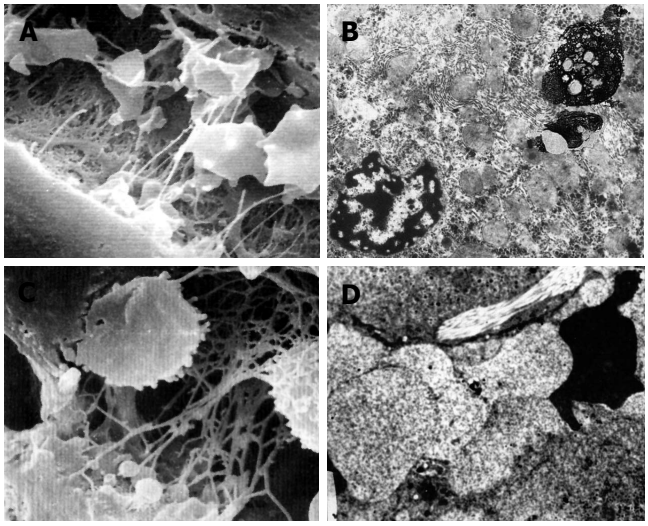


**Figure 3** Cytoplasmic blebs and irregular endothelial sieve plate in some sinusoids after reperfusion (scanning electron microscope × 6000) in the group undergoing warm ischemia injury for 45 min (**A**); apoptosis and necrosis of hepatocytes after reperfusion (× 8000) in the group undergoing warm ischemia injury for 60 min (**B**); cytoplamic blebs, reticular cellulose and hemocytes after reperfusion (scanning electron microscope × 6000) in the group undergoing warm ischemia injury for 60 min (**C**); bleb or ballooning like swollen endothelial cells, blocked sinusoids and irreversible microcirculation disturbance after reperfusion (× 5000) in the group undergoing warm ischemia injury for 60 min (**D**).

degenerated, and rough endoreticule was broken, apoptosis of hepatic and endothelial cells was increased with chromosome margination, karyopyknosis and karyorrhexis in the group undergoing warm ischemia injury for > 30 min (Figures 2C and D). Endothelial gaps were enlarged, the sieve plate was mingled, some endothelial cells broke off, and therefore, some sinusoids were blocked with cytoplasmic bleb accumulation in the groups undergoing warm ischemia injury for 45 and 60 min (Figure 3A). Forty-eight hours after reperfusion, swollen mitochondria resumed gradually, glycogen particles increased obviously in the group undergoing warm ischemia injury for < 45 min. Mitochondria were extended with crista turbulence, vacuole, membrane rupture, and cell apoptosis and necrosis (with karyopyknosis, karyorrhexis and karyolysis) could be noted in the group undergoing warm ischemia injury for 60 min. The above changes were irreversible (Figure 3B). Most endothelial cells underwent necrosis and shedding, many hepatic sinusoids were full of cytoplasmic blebs, reticular fibrosis and hemocytes (Figure 3C). Under scanning electron microscope, endothelial cells presented with bleb or ballooning like degeneration, sinusoids were blocked, thus the microcirculation underwent irreversible disturbance (Figure 3D).

### *Histochemical observation*

Our previous study has demonstrated that hepatic glycogen begins to reduce when WIT is prolonged to 30 min and the activities of SDH, CO and ATPase begin to decrease when WIT is longer than 45 min[10]. The above changes were positively related to WIT in a time dependent manner during reperfusion in this study. PAS reaction and enzyme activities showed a recovering potency in the groups undergoing warm ischemia injury for 15 and 30 min. The liver graft underwent an irreversible injury and no evident recovery potency was found after implantation in the groups undergoing warm ischemia injury for 45 and 60 min.

## DISCUSSION

How to evaluate the quality of liver grafts and how to ascertain the safety time limit for warm ischemia of liver grafts remain to be solved since warm ischemia injury affects the outcome of liver transplantation. Western transplantation community does not consider much of warm ischemia injury because their liver grafts are mainly taken from "brain-death" donors. Liver transplantation has become an effective management in the treatment of end-stage liver diseases, while the shortage of donor liver is a critical limiting factor for liver transplantation, thus people have begun to reconsider the marginal organ source like NHBD since 1990s[11-13]. Some laboratory studies support a controversial 60 min WIT limitation[14-16], but different experimental animals, warm ischemia models and experiment conditions may cause arguments about the WIT limitation. The argument about the WIT limitation has led to a worldwide investigation on warm ischemia-reperfusion injury[17,18].

In the present study, we observed the changes of histological structure and ultrastructure of liver grafts during different WIT. Cellular edema and vacuole degeneration could be noted in warm ischemia period. The pathologic changes were aggravated with the prolongation of WIT, but necrosis was absent. Hepatocyte vacuole degeneration was due to swollen mitochondria and outstretched endoresticule. Glycogen-absorbed vacuole also could be seen. Under electron microscope, pathologic

changes were reversible, only part of cells underwent irreversible changes such as necrosis (with karyopyknosis, karyorrhexis and karyolysis) and apoptosis. However, liver grafts from NHBD underwent injuries both in the warm ischemia period and in the reperfusion stage. The degree of injury in the reperfusion stage was positively related to the duration of WIT. Histochemical observations showed that hepatic glycogen began to reduce when WIT was prolonged to 30 min. The activities of SDH, CO and ATPase began to decrease when WIT was longer than 45 min. The above changes were positively related to WIT in a time-dependent manner during the reperfusion period. PAS reaction and enzyme activities showed a recovering potency in the groups undergoing warm ischemia injury for 15 and 30 min. The liver graft underwent irreversible injury and no evident recovery potency was found after implantation in the groups undergoing warm ischemia injury for 45 and 60 min.

In conclusion, the pathologic changes of liver grafts undergoing only warm ischemia injury are reversible when WIT is shorter than 60 min, but the damage to liver graft would aggravate at the reperfusion stage, suggesting that rat liver grafts undergoing warm ischemia injury are in the reversible stage when WIT is within 30 min. The 45 min of WIT may be a critical point of rat liver grafts to tolerate warm ischemia injury and when WIT is prolonged to 60 min, the damage is irreversible.

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**S- Editor** Wang J **L- Editor** Wang XL **E- Editor** Bi L

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*World J Gastroenterol* 2006 August 14; 12(30): 4906-4907
***World Journal of Gastroenterology*** ISSN 1007-9327


*CASE REPORT*

# Alpha-1-antitrypsin deficiency resulting in a hitherto unseen presentation of hepatocellular carcinoma: Polycythemia but with normal alpha fetoprotein

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Received: 2006-03-17 Accepted: 2006-04-21

## Abstract

Polycythemia is a known paraneopastic manifestation of hepatoma, but only in the presence of alpha-fetopro (AFP). We present a case of polycythemia in the absence of AFP, and suggest concurrent alpha-1-antitrypsin deficiency as the cause for breaking this rule. We also suggest a reason for the apparent constant conjunction between polycythemia and AFP in hepatoma.



**Key words**: Hepatoma; Polycythemia; Alpha 1 anti trypsin; Ephrin-A; Alpha-fetopro

Owen DR, Sivakumar R, Suh ES, Seevaratnam M. Alpha-1-antitrypsin deficiency resulting in a hitherto unseen presentation of hepatocellular carcinoma: Polycythemia but with normal alpha fetoprotein. *World J Gastroenterol* 2006; 12(30): 4906-4907

http://www.wjgnet.com/1007-9327/12/4906.asp

## INTRODUCTION

Polycythemia is a known paraneopastic manifestation of hepatoma, but only in the presence of alpha-fetopro (AFP). We present a case of polycythemia in the absence of AFP, and suggest concurrent alpha-1-antitrypsin deficiency as the cause for breaking this rule. We also suggest a reason for the apparent constant conjunction between polycythemia and AFP in hepatoma.

## CASE REPORT

A 79-year-old man was referred for dizziness with a history of benign prostatic hypertrophy and cholecystectomy. He was taking finasteride and ranitidine. He was an ex-smoker with a forty-pack year history and had never drunk alcohol heavily. Clinical examination was unremarkable but for a smoothly enlarged liver. Full blood count revealed polycythemia (haemoglobin 176 g/L, haematocrit 0.527), with a normal platelet count and white cell count. Chest X ray and ECG were normal. Oxygen saturation was 96% on air.

Abdominal ultrasound revealed a normal spleen and a 47 mm × 57 mm × 49 mm mass in the right lobe of the liver posteriorly, which was compressing the inferior vena cava. A CT scan demonstrated a well-defined rounded mass in the hilum of the liver with a mixed attenuation pattern, consistent with either hepatocellular carcinoma (HCC) or haemangioma. Serum AFP [8 μg/L (normal 0-16)], liver function tests, and hepatitis serology were normal, except IgG antibodies to hepatitis A. MRI of the liver suggested HCC as a more likely diagnosis than haemangioma. With normal AFP and liver function tests, a biopsy of the lesion was carried out. The histology confirmed HCC with adjacent cirrhosis.

He was referred to a specialist centre and further imaging confirmed extra hepatic disease with intra-abdominal and mediastinal lymphadenopathy. There was local vascular invasion but no peritoneal dissemination. A further liver biopsy found underlying fibrosis and DPAS (diastase periodic acid Schiff) positive globular material in the hepatocytes, consistent with alpha-1-antitrypsin (AAT) deficiency. Because of vascular involvement he was deemed unsuitable for surgery and was entered into a trans-arterial embolisation versus chemoembolisation trial.

## DISCUSSION

Hepatocellular carcinoma can present with various paraneoplastic manifestations including polycythemia, hypercholesterolemia, hypoglycemia and hypercalcemia[1]. Our case is unique as it demonstrates an unreported phenomenon: HCC with polycythemia, but normal serum AFP. Polycythemia is strongly related to tumour burden and AFP, and is usually associated with markedly raised serum AFP levels[2,3].

Polycythemia is only partly due to increased erythropoietin production, as raised serum erythropoietin can be present in up to 23% of HCC patients[4], yet polycythemia is found only in approximately 1% of patients. This implies that erythropoietin production may be necessary, but is certainly not sufficient for polycythemia, and other factors must be implicated.

One such factor may be the expression of the erythropoietin receptor. This is upregulated by Ephrin-A1, a ligand for the Eph (erythropoietin producing hepatocellular) receptor tyrosine kinase[5]. Ephrin-A1 expression upregulating the erythropoietin receptor and thus resulting in the appearance of polycythemia would explain the constant conjunction hitherto reported in HCC between polycythemia and AFP, as there is a strong correlation between the presence of AFP in HCC with the expression of Ephrin-A1, which is known to induce AFP[5].

We therefore suggest that the association between polycythemia and raised AFP previously noted in HCC is because both arise from the expression of Ephrin-A1.

Our patient's normal AFP (despite his polycythemia) may be related to his AAT deficiency. Previous reports demonstrated high serum AFP levels in neonates with neonatal hepatitis, either idiopathic or due to extrahepatic biliary atresia. However, AFP is not raised in those infants with neonatal hepatitis and AAT deficiency. It was postulated that this is because alpha-1-antitrypsin is a rate limiting factor in the production of AFP[6]. To our knowledge, the possibility of AAT deficiency resulting in normal AFP in HCC in adults has not been raised.

## ACKNOWLEDGMENTS

With thanks to Professor Roger Williams CBE, Institute of Hepatology, The Royal Free and University College Medical School, Gower Street, London, WC1E 6BT. UK.

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**S- Editor** Pan BR **L- Editor** Zhao JB **E- Editor** Bai SH

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*World J Gastroenterol* 2006 August 14; 12(30): 4908-4910
***World Journal of Gastroenterology*** ISSN 1007-9327


*CASE REPORT*

# Retinal vein thrombosis associated with pegylated-interferon and ribavirin combination therapy for chronic hepatitis C

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Received: 2006-03-13 Accepted: 2006-04-21

## Abstract

An estimated 300 million people worldwide suffer from chronic hepatitis C with a prevalence of 0.8%-1.0% of the general population in Canada. An increasing pool of evidence exists supporting the use of pegylated-interferon (pegIFN) and ribavirin combination therapy for hepatitis C. We report a 49-year old male of North American aboriginal descent with chronic hepatitis C (genotype 2b). Biopsy confirmed that he had cirrhosis with a 2-wk history of left eye pain and decreased visual acuity. He developed retinal vein thrombosis after 16 of 24 wk of pegIFN-α 2a and ribavirin combination therapy. He was urgently referred to a retinal specialist and diagnosed with non-ischemic central retinal vein occlusion of the left eye. PegIFN and ribavirin combination therapy was discontinued and HCV RNA was undetectable after 16 wk of treatment. Hematologic investigations revealed that the patient was a factor V Leiden heterozygote with mildly decreased protein C activity. Our patient had a number of hypercoagulable risk factors, including factor V Leiden heterozygosity, cirrhosis, and hepatitis C that alone would have most likely remained clinically silent. We speculate that in the setting of pegIFN treatment, these risk factors may coalesce and cause the retinal vein thrombosis.



**Key words**: Interferon; Pegylated-interferon; Hepatitis C; Cirrhosis; Retinal vein thrombosis; Thrombosis; Central retinal vein occlusion

Zandieh I, Adenwalla M, Cheong-Lee C, Ma PE, Yoshida EM. Retinal vein thrombosis associated with pegylated-interferon and ribavirin combination therapy for chronic hepatitis C. *World J Gastroenterol* 2006; 12(30): 4908-4910

http://www.wjgnet.com/1007-9327/12/4908.asp

## INTRODUCTION

An estimated 300 million people worldwide suffer from chronic hepatitis C with a prevalence of 0.8%-1.0% of the general population in Canada[1]. In the last 10 years, dramatic advances have been made in the treatment of this common chronic condition. The pegylated-interferon (pegIFN) and ribavirin combination therapy has been shown to result in sustained virologic response rates of 46%-77%, depending on viral genotype[2]. Evidence has also emerged regarding the utility of interferon in cirrhotic hepatitis C treatment with reduced rates of both hepatocellular carcinoma and improved survival[3-5]. With the growing enthusiasm amongst patients and physicians alike, in favour of treatment as a result of the increasing pool of evidence supporting the use of interferon-based regimens, its adverse effects need to always be recognized and periodically reviewed.

Although interferon or pegIFN therapy can affect any organ system, the most commonly reported side effects include flu-like symptoms such as fever, chills, myalgia, fatigue, diarrhea, nausea and vomiting. Central nervous system disturbances including depression, suicidal ideation, confusion and mental status changes can occur, especially in patients with pre-existing histories. Hematologic side effects, including anemia, thrombocytopenia, and neutropenia, require ongoing monitoring. The reported withdrawal rates due to adverse effects, in studies examining interferon-based combinations are 7%-8%[2,6].

We report a case of central retinal vein thrombosis in a cirrhotic hepatitis C patient during pegIFN and ribavirin combination treatment.

## CASE REPORT

A 49-year old male of North American aboriginal descent, with chronic hepatitis C (genotype 2b) and biopsy confirmed cirrhosis, presented with a 2-wk history of left eye pain and decreased visual acuity, after a 16-24 wk course of therapy with pegIFN-α 2a at a dose of 180 μg per week injected subcutaneously and 800 mg ribavirin per day (Pegasys, Hoffmann-La Roche, Mississauga, ON, Canada). His past medical and family histories were negative for any thrombophilia. Specifically, he had no

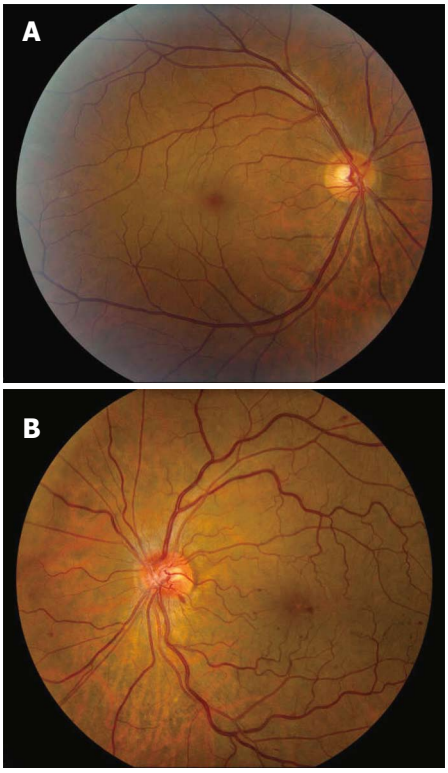


**Figure 1** Normal fundus in the right eye (**A**) and marked venous tortuosity and scattered retinal hemorrhages in the left eye (**B**).

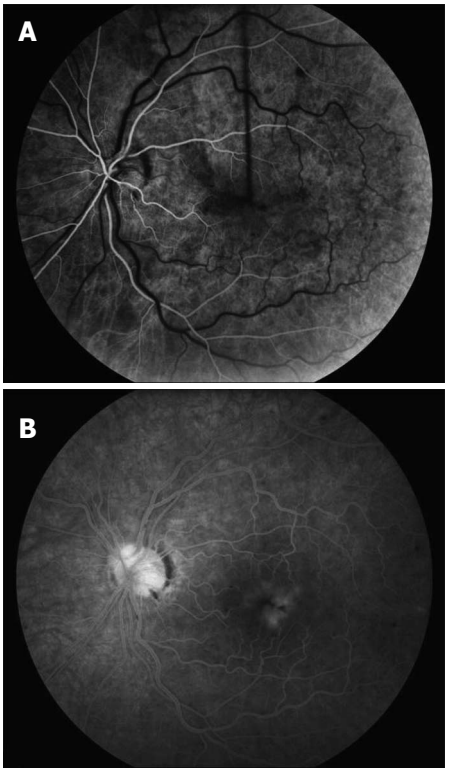


**Figure 2** Left eye angiogram. **A**: Early phase image revealing delayed venous filling; **B**: Late phase image revealing leakage of fluorescein in the macula.

history of superficial or deep venous thrombosis and no history of thromboembolic events. Prior to the initiation of treatment, he had no evidence of decompensated liver disease and his serum alanine aminotransferase (ALT) was 256 U/L (upper limit of normal < 50U/L). Abdominal sonographic imaging revealed a cirrhotic liver with mild splenomegaly. Serial blood work performed at 4 and 8 wk of treatment revealed that his serum ALT levels were 67 U/L and 45 U/L (normal < 55 U/L), respectively. There were no complications associated with the treatment regimen prior to his presentation at 16 wk. One week following the onset of left eye pain and decreased visual acuity, he was assessed by an optometrist who prescribed eyeglasses. Due to the continued symptoms he presented to our hepatitis clinic, two weeks after the initial onset of symptoms. He was urgently referred to a retinal specialist and diagnosed with non-ischemic central retinal vein occlusion of the left eye (Figure 1). Fluorescein angiogram revealed delayed venous filling (Figure 2A) and associated macular edema (Figure 2B). Visual acuity at presentation was 20/20 in the right eye, and 20/70 in the left eye. PegIFN & ribavirin combination therapy was discontinued and the HCV RNA after 16 wk of treatment was undetectable.

Subsequent hematologic investigations to look for a hypercoagulable condition revealed heterozygosity for factor V Leiden, and a mildly decreased protein C activity at 0.49 U (lower limit of normal 0.65 U), confirmed by repeat testing. Antithrombin III and protein S levels were within normal limits but at the lower range with values of 0.72 U and 0.66 U, respectively (lower limits of normal for antithrombin III and protein S, 0.70 and 0.65 respectively). Cryoglobulins and the lupus anticoagulant were negative. Six months following the onset of our patient's left eye pain, his visual deficit remained unchanged.

## DISCUSSION

Interferons comprise a group of pleiotropic proteins with anti-viral, anti-inflammatory, and anti-angiogenesis characteristics. Interferons are also multifunctional immunoregulatory cytokines with effects at various points in the cytokine cascade, likely accounting for their immunostimulatory effects[7]. Due to their various mechanisms of action, interferons are well recognized to cause a variety of side effects as previously mentioned. From an ophthalmologic standpoint, there have been few reported cases of retinal vein thrombosis in patients treated with interferon or pegIFN. We are aware of only three reports in the medical literature[8-10]. Of these, two of the reports describe this rare complication in cirrhotic patients being treated for hepatitis C[9,10].

Regarding the potential thrombogenic properties of interferon, Guyer *et al*[11] in a 1993 paper reporting retinopathy, have suggested that IFN therapy may cause immune complex deposition in the retinal vasculature and leukocyte infiltration, leading to retinal ischemia, congestion, and hemorrhage. Interferon therapy has also been reported to induce a number of thrombogenic autoantibodies, including cryoglobulins, anti-nuclear, anti-smooth muscle, anti-liver-kidney microsomal, anti-thyroglobulin, and anti-phospholipid antibodies, which are thought to play a role in the pathogenesis of a hypercoagulable state[10].

In our patient, the causative role of pegylated-interferon therapy in inducing a hypercoaguable state that results in the retinal vein occlusion is strong given the temporal occurrence. However other risk factors may also have contributed. Considering the key role of the liver in coagulation, cirrhosis results in various impairments via multiple mechanisms: quantitative and qualitative

platelet defects, decreased production of coagulation and inhibitor factors, vitamin K deficiency, synthesis of abnormal clotting factors, decreased clearance of activated factors by the reticuloendothelial system, hyperfibrinolysis, and disseminated intravascular coagulation. The natural inhibitors of coagulation, antithrombin Ⅲ, protein C and protein S, were at low to borderline levels of activity in our patient. Nevertheless, it is important to note that plasma activity of inhibitors between 50% and 70% alone, is not associated with increased thrombotic events in cirrhotics, possibly because of the proportional impairment of procoagulants[12].

As part of the post diagnostic thrombophilic workup, our patient was found to be a heterozygote for the factor V Leiden mutation. Normally, factor V circulates in plasma as an inactive cofactor, awaiting activation by thrombin. Its inactivation requires protein C-mediated cleavage at arginine 306 and arginine 679. Genotypically, a point mutation in the gene-encoding factor V results in a missense mutation. The gene product, called factor V Leiden, which is not susceptible to cleavage by activated protein C, is inactivated more slowly and therefore confers an increased risk of venous thrombosis. The prevalence of heterozygosity for factor V Leiden is 5%-6% and is the most common inherited thrombophilia[13]. The lifetime risk of thrombosis in heterozygotes compared to patients with no defect has been found to range 2.2-4.9[14,15].

The hepatitis C virus itself has been found to induce a variety of potentially thrombogenic antibodies such as cryoglobulins, anti-nuclear, anti-smooth muscle, anti-cardiolipin and anti-phospholipid antibodies[16]. Since the envelope proteins of cytomegalovirus and herpes viruses have been reported to function as a source of procoagulant phospholipid, one could speculate that the hepatitis C envelope could also have procoagulant activity[17].

Our patient had a number of hypercoagulable risk factors that alone, most likely would have remained clinically silent. We speculate that in the setting of pegIFN treatment, these risk factors may coalesce and result in the retinal vein thrombosis. It is interesting that the three cases of retinal vein thrombosis described by Nadir *et al*[10] were being actively treated for hepatitis C with pegIFN, but were also found to have primary defects contributing to a hypercoagulable state including protein S deficiency, hyperhomocysteinemia, heterozygosity for factor V Leiden, anti-phospholipid and anti-cardiolipin antibodies. Therefore we conclude that pegIFN treatment is an important risk factor for the development of retinal vein thrombosis, however based on our case and those described in the literature, other underlying risk factors may also need to be present. We emphasize that retinal vein thrombosis is still a rare complication, and we would not advocate the routine thrombophilic work-up of patients being considered for pegIFN treatment. However, the diagnosis needs to be considered in any patient on pegIFN presenting with decreased visual acuity or eye pain and any patient on pegIFN therapy presenting with manifestiations of a thrombotic episode needs to undergo further hematologic investigation.

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**S- Editor** Wang J **L- Editor** Wang XL **E- Editor** Bai SH

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*World J Gastroenterol* 2006 August 14; 12(30): 4911-4913
***World Journal of Gastroenterology*** ISSN 1007-9327


*CASE REPORT*

# Solitary pulmonary metastasis arising thirteen years after liver transplantation for HBV-related hepatocellular carcinoma

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Received: 2004-08-11 Accepted: 2004-12-14

## Abstract

We described a 59-year-old male patient who underwent liver transplantation in 1989 for hepatocellular carcinoma (HCC) complicating hepatitis B virus (HBV) cirrhosis. In 2001 (12 years after liver transplantation), he developed a lung metastasis of HCC without intrahepatic recurrence and the resection was done. In July 2003, he was symptom free without any recurrence. HCC metastasis can develop even after a very long time of liver transplantation. Many HCCs grow slowly, and the growth rate of recurrent tumors in patients receiving immunosuppressive therapy is significantly greater than that of those who do not receive immunosuppressive therapy.



**Key words:** Hepatitis B virus; Liver transplantation; Hepatocellular carcinoma; Metastasis; Immuno-suppression

Viola C, Asselah T, Samuel D, Durand F, Boudjema H, Valla D, Marcellin P. Solitary pulmonary metastasis arising thirteen years after liver transplantation for HBV-related hepatocellular carcinoma. *World J Gastroenterol* 2006; 12(30): 4911-4913

http://www.wjgnet.com/1007-9327/12/4911.asp

## INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common cancer in the world and the third cause of cancer-related death[1]. Cirrhosis mainly caused by hepatitis B and C viruses (HBV and HBC) constitutes the main risk factor for HCC with an yearly cumulative incidence of 3%[1].

Liver transplantation is claimed to simultaneously cure the tumor and the underlying cirrhosis in selected patients. The 5-year survival rate can be achieved in 75% of optimal candidates for liver transplantation (single nodule < 5 cm or up to three nodules < 3 cm in diameter) with a recurrence rate below 15%[1,2]. For patients with a single resectable HCC complicating cirrhosis, an alternative strategy is to offer resection first and then liver transplantation, if the tumor recurs or if the liver function deteriorates (salvage OLT)[3].

However, the rate of HCC recurrence is high even after liver transplantation[4]. The most frequent sites of recurrence of HCC after OLT are the lung (with a frequency of 51%) and the liver allograft (46%)[4,5]. The great majority of cancer recurrences appear within 5 years of liver transplantation.

We described a 59-year-old male patient who underwent liver transplantation in 1989 for HCC complicating HBV cirrhosis. In 2001 (12 years after liver transplantation), he developed a lung metastasis of HCC without intrahepatic recurrence and the resection was done. In July 2003, he was symptom free without any recurrence.

## CASE REPORT

A 59-year-old man developed deep fatigue and jaundice in May 1986. His serum alanine aminotransaminase was 145 IU/L (normal < 40 IU/L) and tests for serum HBsAg, HBcAb, and HbeAb were positive, HBV-DNA was undetectable by hybridization assay. A liver biopsy showed cirrhosis. Serum level of alpha-feto-protein was 200 ng/mL (normal ⩽ 15 ng/mL). Imaging studies (computerized tomography and ultrasound) showed a nodule (2 cm in diameter) in segment V with typical features of HCC.

In February 1987, segment V was resected. Histological examination confirmed a 2-cm nodule of HCC with a pseudo-capsule characterized by a sclerotic tissue with an intravascular extension of the tumor. After the intervention, alpha-feto-protein decreased to 20 ng/mL.

In December 1988, alpha-feto-protein raised to 100 ng/mL. Ultrasound and arteriography revealed nodules in the segments Ⅳ and Ⅷ. The patient received three cures of chemoembolization (from December 1988 to March 1989) and 3 MU interferon thrice a week for 3 mo until liver transplantation, in order to make serum HBV-DNA negative. In August 1989, orthotopic liver transplantation was performed. Histological examination of the implanted liver showed micronodular cirrhosis without any evidence of neoplasia. He was given cyclosporin (18 mL/d),

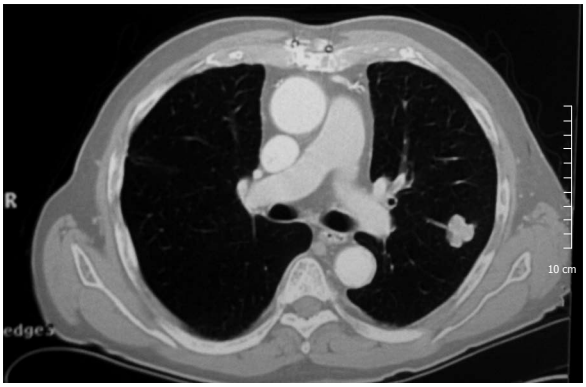


**Figure 1** A 2-cm polylobulated and homogeneously dense lung nodule in the upper left lobe with no calcifications.

prednisone (20 mg/d) and azathioprine (150 mg/d). During the following 2 mo (October 1989), he was treated with cyclosporin (10 mL/d), prednisone (15 mg/d), and azathioprine (50 mg/d). During the following 14 mo (October 1990), he was treated with cyclosporin (0.6 mL/d), prednisone (10 mg/d), and azathioprine (50 mg/d).

During the following years, he had a 4-mo evaluation. Transaminase, alpha-feto-protein and imaging studies (ultrasound and thoraco-abdominal CT) did not show any sign or symptom of HCC recurrence. Rejection and opportunistic infections did not occur. He did not receive HBsAb immunoglobulins and had no reactivation of HBV infection.

In July 2001 (143 mo after liver transplantation), CT scan revealed a 2-cm isolated nodule in the upper left lung lobe, which was polylobulated and homogeneous without calcification (Figure 1). Alpha-feto-protein increased for the first time to 105 ng/mL. He was treated with prednisone (4 mg/d) and azathioprine (50 mg/d). In May 2002 (153 mo after liver transplantation), an atypical resection of the upper left lobe was performed. Histological examination showed poorly differentiated HCC metastasis. After the intervention, alpha-feto-protein returned to its normal level (3.5 μg). Repeated biochemical and imaging examinations did not show any recurrence in the lung, liver or other organs.

At the last follow-up visit (167 mo after liver transplantation and 14 mo after lung resection), he was symptom free. Thoraco-abdominal CT scan did not show any recurrence (July 2003) and serum alpha-feto-protein was 2 ng/mL (normal ⩽ 15 ng/mL).

## DISCUSSION

Recombinant interferon-α (IFN-α) treatment prior to liver transplantation does not seem to reduce the rate of HBV infection; a residual infectivity may persist even in the absence of detectable serum HBV-DNA by standard method and its role in the carcinogenetic process cannot be eliminated[6].

The 5-year survival rate can be achieved in 75% of optimal candidates for liver transplantation (early HCC, single nodule < 5 cm or up to three nodules < 3 cm in diameter) with a recurrence-free survival rate of 92%[7]. Most recurrent tumors arise during the first 2 years after resection, which might be explained by the multicentric nature of HCC in cirrhotic livers rather than by intrahepatic metastasis. The multifocal nature of HCC was examined in the livers from patients undergoing liver transplantation. Certain histological findings in the implant, such as the presence of capsular and microvascular invasion, are considered as signs of a more aggressive tumor associated with a greater incidence of recurrence[8].

**Table 1 HCC recurrence after liver transplantation**

| | Number of patients | Recurrence (%) | Pulmonary metastasis (%) | Mean delay (mo) |
|---|---|---|---|---|
| Yokoyama, 1991[9] | 100 | 43 (43/100) | 12 (12/100) | 34 |
| Ferris, 1996[4] | 124 | 28 (35/124) | 14 (18/124) | 18 |
| Wallis March, 1997[10] | 214 | 40 (71/214) | 11 (23/214) | 24 |
| Total | 438 | 34 (149/438) | 12 (53/438) | 25 |

When recurrence after liver transplantation occurs, the most frequent sites are lungs (51%), liver allograft (46%), and lymph nodes (43%). Ferris *et al*[4] have reported a mean interval of 18 mo.

In the three major studies[4,9,10] involving HCC, the recurrence rate is 34% in 438 patients after liver transplantation (Table 1).

In a great majority of cases, recurrence of HCC occurs within 5 years of liver transplantation. The longest time to recurrence described in the literature is 124 mo[4]. To our knowledge, the time between liver transplantation and recurrence is the longest in our case (12 years, 143 mo).

An active approach to the management of resectable pulmonary metastasis from HCC is justified in selected patients, which can permit a prolonged survival[11]. Most pulmonary metastases of HCC are multiple and not amenable to surgical resection. If any solitary pulmonary metastasis encountered is resectable, the patient should undergo surgery.

The selection of patients with early HCC is the main factor affecting HCC recurrence after liver transplantation. At this early stage of tumor development, there are no other factors that have prognostic values. In patients from Western countries, the progression of HCC is usually slow and is related to tumor size[2]. The mechanism for late recurrence of HCC remains unclear and some hypotheses have been proposed such as intraoperative surgical manipulation[12], embolization of tumor cells via the hepatic veins before or during liver transplantation, which can result in the trapping of micrometastasis within the capillary network of the lungs and immunosuppressive therapy potentiating macroscopic growth of nodules[5].

The effect of long-term immunosuppressive therapy on tumor growth in patients with HCC is unknown. It has been suggested that while many HCCs are growing slowly, the growth rate of recurrent tumors in patients receiving immunosuppressive therapy is significantly greater than that in those who do not receive immunosuppressive therapy, indicating that immunosuppressive therapy plays a major role in tumor recurrence after liver transplantation[9].

In fact, it has been shown that the risk of recurrence in patients who continue to receive corticosteroids may be as much as four times higher than that in patients who stop receiving corticosteroids soon after the liver transplantation[2,6].

In our case, immunosuppressive therapy seemed to be well balanced, because the patient had neither rejection nor opportunistic infections.

In conclusion, HCC metastasis can develop even after a long time of liver transplantation. A systematic long-term follow-up is necessary. In case of single lung metastasis without any other localization, it is possible to resect it, allowing to prolong the survival of the patient. It is very important to maintain vigilance after liver transplantation, because the risk of recurrence exists for a long time after liver transplantation.

Immunosuppressive therapy, one of the possible key factors in controlling the response to neoplasm, can reduce the treatment time[13] and achieve immunologic tolerance and reduce the use of immunosuppressive drugs[12].

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**S- Editor** Wang J **L- Editor** Wang XL **E- Editor** Liu WF

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*World J Gastroenterol* 2006 August 14; 12(30): 4914-4917
***World Journal of Gastroenterology*** ISSN 1007-9327


*CASE REPORT*

# Biliary tuberculosis causing cicatricial stenosis after oral anti-tuberculosis therapy

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Received: 2006-02-08 Accepted: 2006-03-10

## Abstract

A 36-year-old Philippine woman presented with dark urine and yellow sclera. Endoscopic retrograde cholangiopancreatography (ERCP) confirmed dilatation of the intrahepatic bile ducts and also showed an irregular stricture of the common hepatic duct at the liver hilum. Histological examination of biopsies from the bile duct revealed epithelioid cell granulomas and caseous necrosis. Tubercle bacilli were then detected on polymerase chain reaction (PCR) testing of the bile, giving the diagnosis of biliary tuberculosis. Although microbiological cure was confirmed, the patient developed cicatricial stenosis of the hepatic duct. She underwent repeated treatments with endoscopic biliary drainage (EBD) tubes and percutaneous transhepatic biliary drainage (PTBD) tubes, and the stenosis was corrected after 6 years. We present a case of tuberculous biliary stricture, a condition that requires careful differentiation from the more common malignancies and needs long-term follow-up due to the risk of post-treatment cicatricial stenosis, although it is rare.



**Key words:** Biliary tuberculosis; Obstructive jaundice; Cicatricial stenosis; Polymerase chain reaction

Iwai T, Kida M, Kida Y, Shikama N, Shibuya A, Saigenji K. Biliary tuberculosis causing cicatricial stenosis after oral anti-tuberculosis therapy. *World J Gastroenterol* 2006; 12(30): 4914-4917

http://www.wjgnet.com/1007-9327/12/4914.asp

## INTRODUCTION

The more common benign causes of biliary stenosis are postoperative cicatricial stenosis and complications of chronic pancreatitis, duodenal papillitis, and congenital biliary dilatation, whereas tuberculous lesions, such as tuberculosis (TB) of the biliary lymph nodes, pancreatic TB, and biliary TB are rare. In this paper, we report a case of biliary TB causing obstructive jaundice and cicatricial stenosis after oral anti-tuberculosis therapy.

## CASE REPORT

The patient was a 33-year-old female of Philippine origin. She presented with dark urine, yellow sclera, and malaise. She had lived in Japan for 3 years when she was admitted to our hospital. Her father and brother had a past history of pulmonary TB. She received no past treatment for TB. When she visited a local doctor in May 1998 for symptoms of dark urine and yellow sclera, she was found to have mild hepatic dysfunction and was thus referred to our hospital with suspected acute hepatitis. Viral, drug-induced, and auto-immune hepatitis were excluded, and she was treated with watchful anticipation as an outpatient. Abdominal ultrasound then revealed dilatation of the intrahepatic bile ducts and multiple intrahepatic hypodense areas, and the patient was admitted to our hospital for further investigation in February 1999. Admission findings included: height 154 cm, weight 54 kg, and body temperature 36.4℃. Her blood pressure was 112/62 mmHg, heart rate was 64 beats/min, and she had a sinus rhythm. Conjunctiva was not anemic or jaundiced. No superficial lymph nodes were palpable. Abdomen was flat and soft. The liver, spleen or masses were not palpable without abdominal pain or tenderness. Full blood examination revealed that she had mild anemia (117 mg/L; normal: 125-170 mg/L) and an elevated erythrocyte sedimentation rate (66 mm/h; normal: <10 mm/h). Serum biochemistry showed elevated biliary enzyme γ-glutamyltranspeptitase (201 IU/L; normal: 12-70 IU/L). Tumor markers CA19-9 (100 KU/L; normal: <37 KU/L) and PIVKA-II (43 AU/L; normal: <10 AU/L) were elevated. Abdominal ultrasonography (US) showed the hepatic parenchyma to be uniform and slightly hypertrophic, with dilatation of the intrahepatic ducts and multiple hypoechoic masses (Figures 1A and B). Abdominal computed tomography (CT) scans confirmed intrahepatic ductal dilatation and multiple hypodense lesions in the liver, some with micro-calcifications. The early contrast phase images showed slight enhancement of the periphery of the lesions, while the late phase images showed uneven enhancement

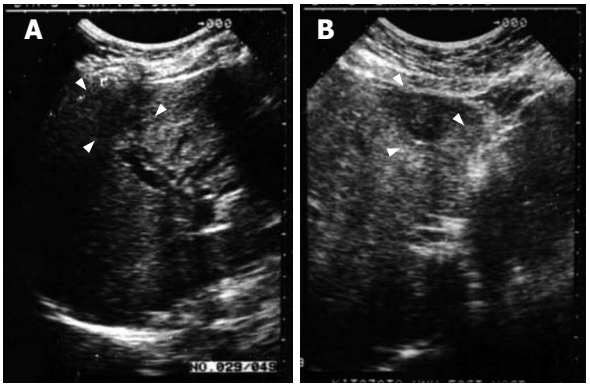


**Figure 1** Ultrasonography findings of the liver. **A**: Dilatation of the intrahepatic ducts and a hypoechoic mass in the right lower anterior segment; **B**: A heterogeneous mass in the inner left segment.

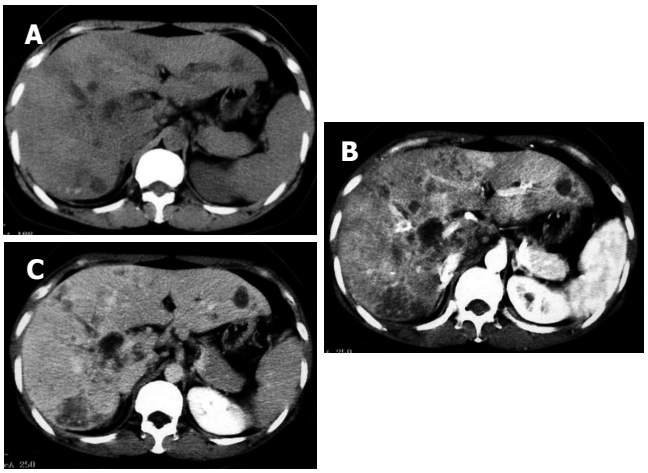


**Figure 2** Abdominal computed tomography (CT). **A**: Plain CT images show intrahepatic ductal dilatation, micro-calcifications, and multiple hypodense lesions in the liver; **B**: Contrast CT image (early phase) shows clearly delineated hypodense lesions; **C**: Late phase CT image shows slightly enhanced hypodense lesions.

of the peripheral and central areas. Lymphadenopathy was seen both at the liver hilum and at the origin of the splenic artery (Figures 2A-C). Intraductal ultrasonography (IDUS) showed soft tissue masses at the liver hilum of the hepatic duct (Figure 3A), and circumferential thickening of the common hepatic duct (Figure 3B). Endoscopic retrograde cholangiopancreatography (ERCP) revealed that the common hepatic duct was narrowed over a 2 cm section, and the hepatic ducts were clumped and irregular at the liver hilum, with strictures of the feeding branches from each section of the liver (Figures 4A and B). Histopathological examination of endoscopic biopsy specimens from the common hepatic duct at the liver hilum revealed granulomas with epithelioid cells (Figure 5A), whereas a biopsy specimen from a hepatic mass showed very mild atrophy and marked dilatation of the hepatic sinuses, with large foci of caseous necrosis surrounded by epithelioid granuloma (Figure 5B). Repeated bile cytodiagnosis showed no malignancy. Cholangiography showed irregular strictures of the intra and extra hepatic biliary ducts. So hepatic secondaries from malignant neoplasia were mostly suspected, but the

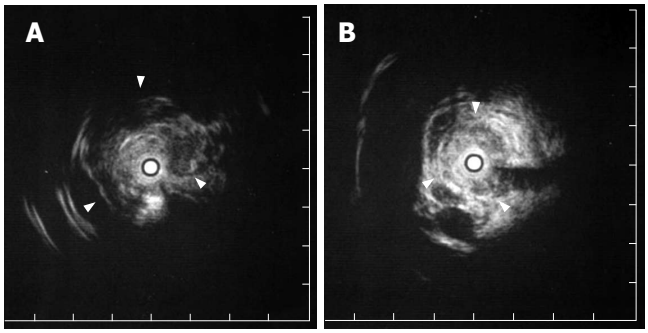


**Figure 3** Endoscopic ultrasonography (EUS). **A**: Soft tissue masses at the liver hilum of the hepatic duct; **B**: Circumferential thickening of the common hepatic duct.

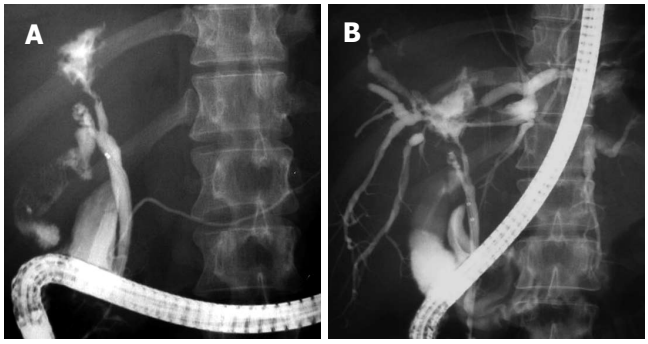


**Figure 4** Endoscopic retrograde cholangiopancreatography examination. **A**: The common hepatic bile duct was narrowed over a 2 cm section, and strictures and irregularities of the hepatic bile duct at the liver hilum were revealed; **B**: The hepatic ducts at the liver hilum were clumped with strictures of the feeding branches from each section of the liver.

biopsies and bile cytodiagnosis did not show malignancy. Then the differential diagnosis could include primary biliary sclerosis (PSC), drug-induced cholestasis, and HIV-associated cholangiopathy. Serum ALP was normal with no obvious elevations in liver enzymes. Also serum anti-mitochondrial antibody and peripheral anti-neutrophil cytoplasmic antibody (pANCA), and smooth-muscle antibody did not elevate. Symptoms of inflammatory bowel disease, particularly ulcerative colitis did not present. She took no medicine and her serum HIV was negative. The biopsy findings of caseous necrosis and epithelioid granulomas, and bile polymerase chain reaction (PCR)-confirmed tubercle bacilli, led to the diagnosis of biliary TB. Tuberculin test was also strongly positive. Triple anti-tuberculosis therapy, comprising 400 mg isoniazid (INH) daily, 750 mg ethambutol (EB) daily, and 450 mg rifampicin (RIF) daily, was administered for 7 mo. Microbiological cure was confirmed in October 1999, with phlegm, gastric juice, bile, and feces negative for *Mycobacterium tuberculosis*. In December 2000, 14 mo after the completion of anti-tuberculosis treatment, the patient became febrile and jaundiced. Endoscopic retrograde cholangiography (ERC) demonstrated cicatricial stenosis of the common hepatic duct at the liver hilum. Because of the tight stricture at the liver hilum, and narrowing of many intrahepatic bile ducts, transpapillary stent placement was abandoned, and percutaneous transhepatic biliary drainage (PTBD) was performed instead (Figure 6A). Although she subsequently

**Table 1 Summary of the 16 previous cases and our case of tubercular biliary stricture**

| No. | Age | Sex | Site of stricture | Initial presentation | Confirmation of diagnosis | Treatment | Outcome | Reference/Nation |
|---|---|---|---|---|---|---|---|---|
| 1 | 30 | M | CBD | CCC | Laparotomy frozen section | T-tube | | Gupta *et al*[2]/India |
| 2 | 78 | F | Multiple | Bacterial cholangitis | Laparotomy frozen section | Laparoscopic cholecystectomy | Died of sepsis | Abascal *et al*[3]/Spain |
| 3 | 46 | F | CHD | CCC | Laparotomy frozen section | PTBD, surgical bypass was abandoned | Post anti-TB therapy, pulmonary calcification | Fan *et al*[4]/Hong Kong, China |
| 4 | 38 | M | CBD | CCC | Laparotomy frozen section | T-tube | | Ratanarapee *et al*[5]/Thai |
| 5 | 46 | F | CHD | | Bile cytology | EBD (Pl, metal) | Biliary stones, restenosis | Bearer *et al*[6]/USA |
| 6 | 40 | M | CBD | CCC | Laparotomy frozen section | Hepaticojejunostomy | | Behera *et al*[7]/India |
| 7 | 45 | F | CBD | CCC | Laparotomy frozen section | Hepaticojejunostomy | | Valeja *et al*[8]/India |
| 8 | 70 | M | CBD, CHD | CCC | Culture of biopsy of inguinal lymph node | ERBD (refused operation) | Post anti-TB therapy, pulmonary calcification | Hickey *et al*[9]/Ireland |
| 9 | 46 | M | CBD | | CT guided FNAB | EBD | Restenosis | Kok *et al*[10]/Brunei |
| 10 | 29 | F | CHD, HD | | Bile cytology | Left cholangiojejunostomy | | Kok *et al*/Brunei |
| 11 | 60 | F | CBD | CCC | Laparotomy frozen section | Open billiary stenting | | Kok *et al*/Brunei |
| 12 | 44 | F | CHD | CCC | Laparotomy frozen section | Hepaticojejunostomy | Hepatic calcification | Kok *et al*/Brunei |
| 13 | 33 | F | CHD | CCC | Laparotomy frozen section | Hepaticojejunostomy | | Yea *et al*[11]/Taiwan, China |
| 14 | 70 | M | HD | | PCR of bile | PTBD | Billroth II reconstruction | Yea *et al* /Taiwan, China |
| 15 | 58 | M | Multiple | CCC | Tissue obtained via PTBD | PTBD (metal) | Beaded type | Inal *et al*[12]/Turkey |
| 16 | 66 | M | CBD, RHD | CCC | Laparotomy frozen section | T-tube, PTCD | Post anti TB therapy | Prasad *et al*[13]/India |
| 17 | 33 | F | CHD | | PCR of bile | PTBD, EBD | Pulmonary calcification, biliary stones, restenosis | Our case/Japan |

CCC: Cholangio cell carcinoma; HD: Hepatic duct, RHD: Right hepatic duct; LHD: Left hepatic duct; CHD: Common hepatic duct; CBD: Common bile duct; FNAB: Fine-needle aspiration biopsy; ERCP: Endoscopic retrograde cholangiopancreatography; PTCD: Percutaneous transhepatic biliary drainage; EBD: Endoscopic biliary drainage.

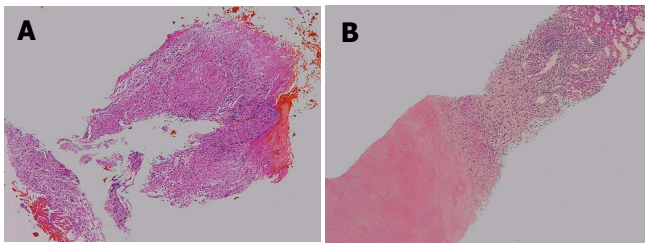


**Figure 5** Histological findings of the biopsy specimen stained with HE. **A**: Photomicrograph of an endoscopic biopsy specimen from the common hepatic duct showing granulomas with epithelioid cells; **B**: Photomicrograph of a biopsy specimen from a hypoechoic mass in the liver showing focal caseous necrosis surrounded by granuloma.

experienced repeated bouts of pyrexia and jaundice due to ascending cholangitis, the stenosis improved gradually, and fistularisation was achieved in July 2001 (Figure 6B). By February 2003, only two 7 Fr pigtail catheters were required for endoscopic biliary drainage (EBD) tube placement (Figure 6C). Two years later (in January 2005), a further bout of cholangitis made the patient febrile but not jaundiced, and no stenoses were detected on ERC (Figure 6D), so the EBD tubes were removed. She developed biliary stones in April of the same year, which was not detected before. However, EBD tube was reinserted and follow-up was continued at the time of writing this paper.

## DISCUSSION

Benign biliary strictures fall into two etiological groups: traumatic (post operative, blunt, or penetrating injury) and nontraumatic (sclerosing cholangitis, recurrent pyogenic cholangitis, chronic pancreatitis, Mirizzi syndrome). The site and number of strictures depend on the cause. TB is a rare cause of biliary obstruction. Hepatobiliary TB may be caused by three ways: spread of caseous material from the portal tracts into the bile ducts (most often), secondary inflammation-related tuberculous periportal adenitis, and spread of caseous material through the ampulla of Vater and ascending along the common bile duct. Hepatobiliary TB can be classified into 3 types: miliary hepatic TB, hepatic tuberculoma, and biliary TB[1]. The majority are the miliary TB type. Hepatic tuberculoma requiring differentiation from hepatoma is relatively rare. Biliary TB is even more uncommon, and no cases of biliary TB causing obstructive jaundice due to biliary stenosis have been reported in Japan. A Pub Med search of papers published after 1985 has yielded 16 reported cases of biliary TB causing obstructive jaundice[2-13] (Table 1). In each case, irregular stenosis of one or more bile ducts was seen on ERC, these findings differing considerably from those in cases of TB of the biliary lymph nodes or pancreatic TB, where obstructive jaundice is caused by extramural compression of the common bile duct (CBD). Differentiation from malignant neoplasia was often extremely difficult, and in 11 of the 16 cases laparotomy was performed without having excluded malignancy, and a preoperative diagnosis of TB was achieved through biopsy or PCR in only 5 cases. In 1 case, although the diagnosis of TB had been made, choledochoduodenostomy was required due to multiple strictures. 2 cases were complicated by biliary stones, and cicatricial restenosis occurred in the same cases following medical treatment. The bile duct might have been severely damaged by repeated inflammatory reactions and have become irreversibly scarred. One case had a postinflamamtory stricture for nearly 2 years[6] and one case required stent changes every 6 mo at the issue[10]. And only 2 cases were

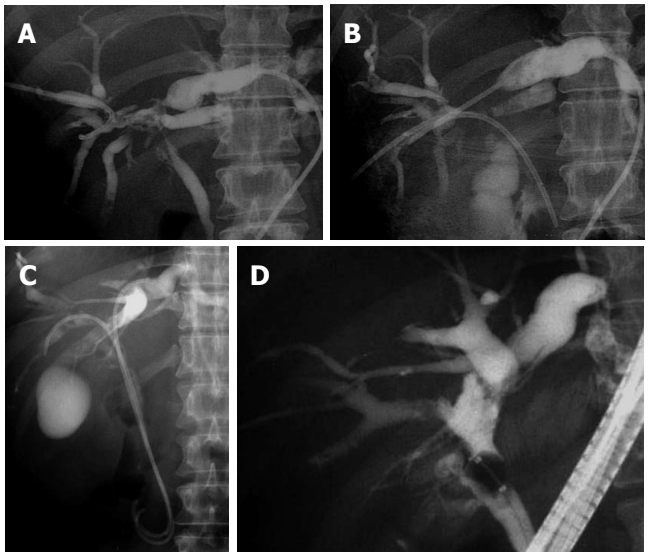


**Figure 6** ERC following anti-tuberculosis therapy showing marked irregularity of the hepatic ducts and strictures of common hepatic duct. **A**: PTBD tubes were inserted via the B8 and B3 branches; **B**: The PTBD tube from the B5 branch was inserted into the common bile duct; **C**: EBD tubes were inserted into both hepatic lobes; **D**: The strictures of the hepatic duct s were recanalized.

with radiological evidence of pulmonary tuberculosis and one case with hepatic calcification, so TB must be considered in the differential diagnosis of any bile duct obstruction, particularly in patients from areas where TB is prevalent.

Biliary TB is a condition with no specific clinical findings and is usually diagnosed through biopsy or the detection of tubercle bacilli. The detection rate through culture is 0%-10%[14]. However, even if epithelioid granulomas are identified, differentiation from conditions such as hepatic sarcoidosis or inflammatory bowel disease is important. Sarcoid granulomas are similar to TB granulomas, although in the former foreign body-type giant cells are seen in addition to Langhans giant cells, and foci of necrosis are rarely seen. In this case, acid-fast bacilli were not detected by culture or microscopy, and *Mycobacterium tuberculosis* was only detected through PCR testing of the bile. Since PCR testing for *Mycobacterium tuberculosis* is extremely sensitive, it should be used extensively. Although favourable results can be achieved by medical therapy with repeated stenting of the bile ducts, long-term follow-up is required due to the risk of post-treatment cicatricial stenosis.

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**S- Editor** Pan BR **L- Editor** Wang XL **E- Editor** Bi L

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*World J Gastroenterol* 2006 August 14; 12(30): 4918-4921
***World Journal of Gastroenterology*** ISSN 1007-9327


*CASE REPORT*

# A case of mucin producing liver metastases with intrabiliary extension

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Received: 2006-03-23 Accepted: 2006-04-21

## Abstract

A 75-year-old man was admitted to our hospital with a diagnosis of liver metastases from colon cancer. He underwent right hemicolectomy for cecal cancer eight years ago, and had a metastatic liver tumor in segment 8 (S8), which was surgically resected about 4 years after the initial operation. Histopathological examination of the resected specimens from both operations revealed a well-differentiated adenocarcinoma with mucinous carcinoma. Four months after the second operation, computed tomography demonstrated a low-density lesion at the cut surface of the remnant liver. Although it was considered to be a postoperative collection of inflammatory fluid, it formed a cystic configuration and increased in size to approximately 5 cm in diameter. With a tentative diagnosis of a recurrence of metastatic cancer, partial hepatectomy of S8 was performed. Histological examination of the resected specimens also revealed mucinous adenocarcinoma, which had invaded into the biliary ducts, replacing and extending along its epithelium. Immunohistochemically, the tumor cells were positive for cytokeratin (CK) 20, but negative for CK7. Therefore, the tumor was diagnosed as a metastatic adenocarcinoma from colonic cancer. Liver metastases of colorectal adenocarcinoma sometimes invade the Glisson's triad and grow along the biliary ducts.



**Key words:** Liver metastases; Mucin; Intrabiliary extension; Cytokeratin 7; Cytokeratin 20

Tokai H, Kawashita Y, Eguchi S, Kamohara Y, Takatsuki M, Okudaira S, Tajima Y, Hayashi T, Kanematsu T. A case of mucin producing liver metastases with intrabiliary extension. *World J Gastroenterol* 2006; 12(30): 4918-4921

http://www.wjgnet.com/1007-9327/12/4918.asp

## INTRODUCTION

Liver metastases can occur in 25%-35% of patients with colorectal carcinoma[1], and surgical resection has become accepted as a reasonable treatment for patients with liver metastases. However, because of their propensity to spread along epithelial surfaces, liver metastases of colorectal carcinoma sometimes invade the Glisson's triad[2]. Therefore, in contrast to hepatocellular carcinoma (HCC), bile duct invasions of liver metastases from colorectal carcinoma are seen in about 10%-12% of resected liver metastases[3,4]. On the other hand, cystic formation caused by mucin producing metastatic tumor from colorectal cancer has only rarely been reported. Here, we report a case of mucin producing liver metastases with intrabiliary extension that was difficult to distinguish from benign cystic change.

## CASE REPORT

A 75-year-old man was admitted to our hospital with a liver tumor. He underwent right hemicolectomy following a diagnosis of cecal cancer in January, 1994. Histological examination of the resected specimens revealed a well-differentiated adenocarcinoma with mucinous carcinoma, which invaded the subserosal layer and metastasized to regional lymph nodes. Four years after his initial operation, he underwent partial hepatectomy of segment 8 (S8) due to a diagnosis of metastatic liver tumor on June 22, 1998. Histopathological examination of the resected specimens revealed a well-differentiated adenocarcinoma with mucinous carcinoma, similar to cecal cancer. The tumor invaded only the bile duct, but surgical margin was negative for cancer. Four months after his second operation, abdominal computed tomography (CT) revealed a low-density lesion at the cut surface of the liver. Initially, it was considered to be a postoperative collection of inflammatory fluid. Through the continuous observation using periodical CT scans, the low-density lesion gradually formed a cystic mass over two years, but little change was found in size. Two years later, the mass increased in size

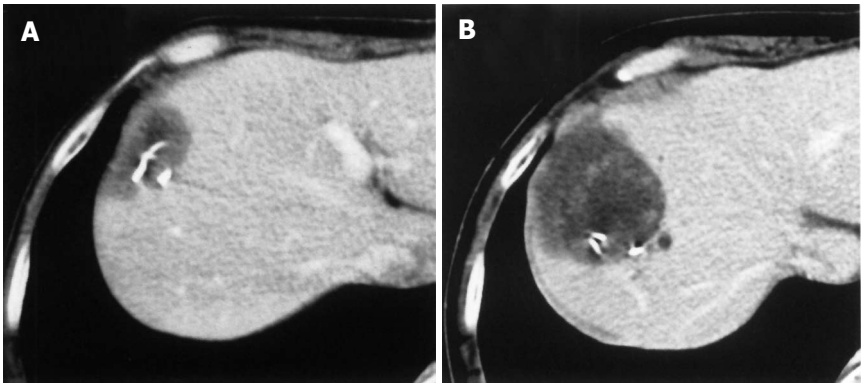


**Figure 1** A low-density lesion at the cut surface of the liver in December, 1998 (**A**) and two years later, the mass increased in size to approximately 5 cm in diameter (**B**).

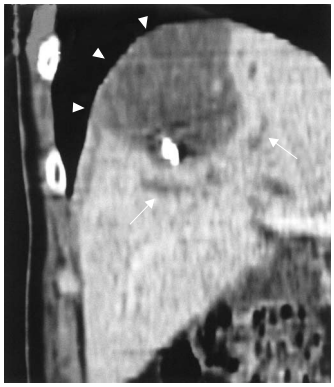

**Figure 2** A low-density cystic tumor in S8 with local dilatation of the IHBD (arrows). The tumor was bordered by the diaphragm (arrow heads).

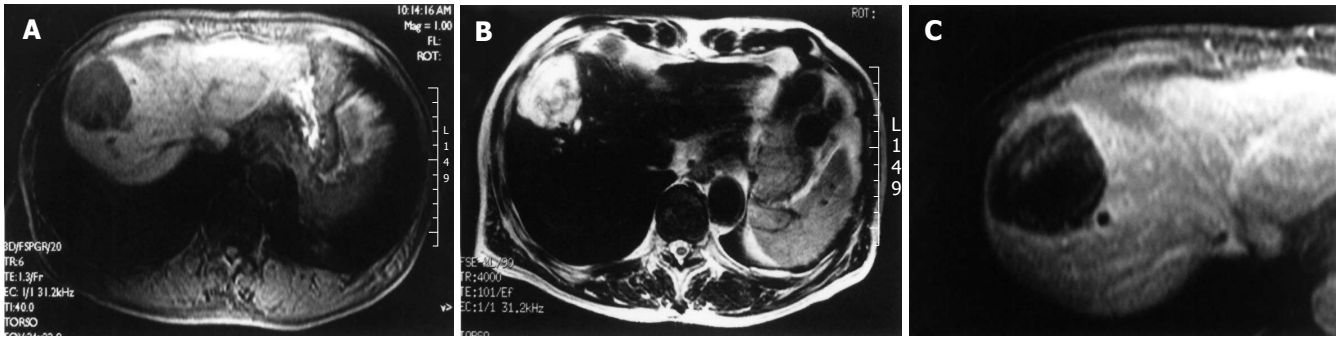


**Figure 3** A low intensity signal on T1-weighted (**A**) and a high intensity signal on T2-weighted (**B**) images. The edge of the tumor was slightly enhanced by gadolinium, while the inside was heterogeneous (**C**).

to approximately 5 cm in diameter (Figure 1). Cytological examination from ultrasonography (US)-guided fine needle aspiration biopsy revealed it to be class III, indicating a borderline malignancy.

On admission, the patient exhibited no abnormal findings upon physical examination. Complete blood counts and serum chemistries were within normal limits. Among tumor markers, serum carcinoembryonic antigen (CEA) levels and alpha-fetoprotein (AFP) levels were within normal limits. The indocyanin green retention rate after 15 min (ICG R15) was 16.0%. Abdominal CT revealed a low- density cystic tumor of S8 that measured about 4 cm × 5 cm in size, with local dilatation of intrahepatic bile ducts (IHBD). The tumor was bordered by the diaphragm (Figure 2). Abdominal magnetic resonance imaging (MRI) revealed a tumor with a low intensity signal on T1-weighted images and a high intensity signal on T2-weighted images (Figures 3A and B). In addition, the edge of the tumor was slightly enhanced by gadolinium, while the inside was heterogeneous (Figure 3C). Magnetic resonance cholangiopancreatography (MRCP) revealed local dilatation of IHBD. Based upon these examinations, one of the differential diagnoses was biloma at the cut end of the previous hepatectomy. To further investigate the content of the tumor, US-guided biopsy was performed. An aspirated sample contained a small volume of yellow and clear fluid, but bile was not found. Histological examination of the biopsy specimens revealed a mucinous adenocarcinoma. Finally, the tumor was judged to be a recurrence of metastatic cancer with invasion to the IHBD. There was no evidence of metastasis in any other organs or in regional lymph nodes. The patient was considered to be a candidate for surgery. On March 13 in 2002, he underwent the third operation. When the peritoneal cavity was entered, there was no evidence of peritoneal dissemination, enlarged lymph nodes or ascites. The tumor was located in the liver in S8 adhering to the diaphragm and was about 4 cm in diameter, as measured by US. US was also performed to indicate a sufficient surgical margin of at least 15 mm. The tumor was resected using intermittent clamping of the primary branch of Glisson's triad (Pringle's procedure). When we cut the regional Glisson's triad, mucinous bile was found at the cut end of the bile duct. The portal vein was intact. According to the preoperative informed consent, partial resection of the liver in sub-segment 8 and the diaphragm was performed. Histological examination of the resected specimens revealed a well-differentiated mucinous adenocarcinoma, which was consistent with the metastatic lesion from colon cancer. The tumor extended along the lumen of the biliary ducts, replacing the non-neoplastic epithelium (Figure 4). The peripheral bile ducts were obstructed by the cancer cells, and the cut end of the bile duct was positive for cancer. The tumor invaded only the abdominal side of the diaphragm and was not apparent on the thoracic side. Immunohistochemically, the tumor

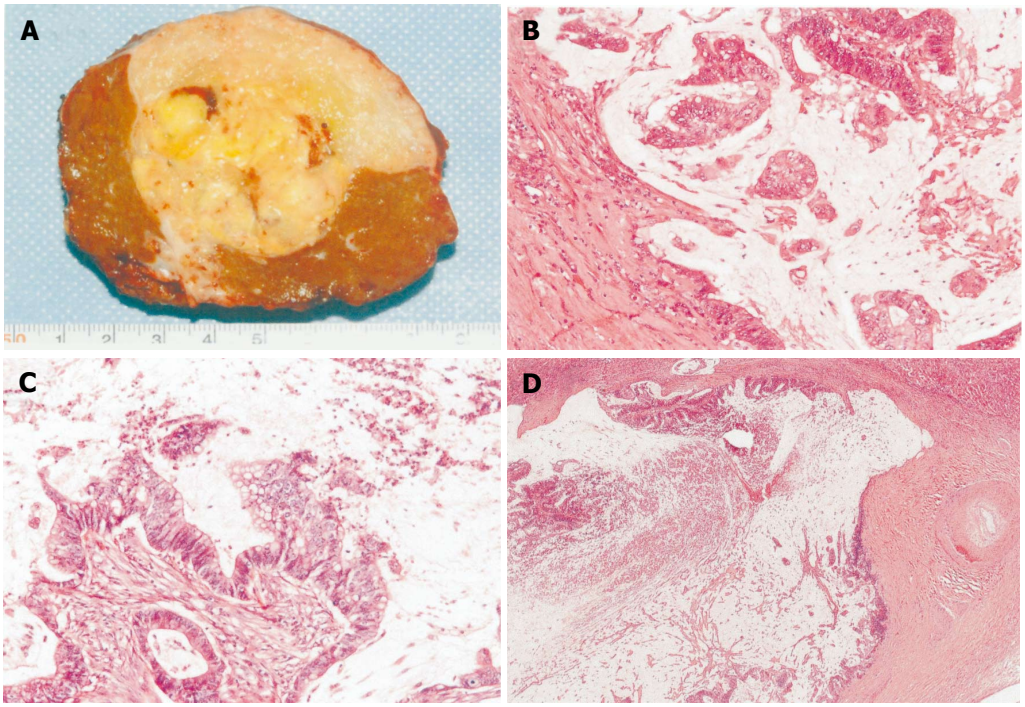


**Figure 4** Macroscopic finding (**A**), well-differentiated mucinous adenocarcinoma (**B**) similar to cecal cancer (**C**), extension of tumor cells along the lumen of the biliary ducts with the non-neoplastic epitheliumreplaced (**D**). (B) HE, × 100; (C) HE, ×100; (D) HE, ×18.

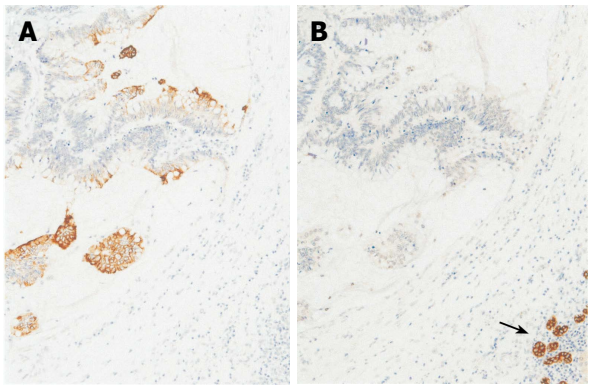


**Figure 5** Immunohisotochemically, tumor cells positive for CK 20 (**A**), but negative for CK7 (**B**), normal epithelial cells of IHBD positive for CK7 (arrow). (**A**) ×100; (**B**) ×100.

cells were positive for CK 20, but negative for CK7 (Figure 5). Thus, we diagnosed it as a recurrence of metastatic adenocarcinoma from cecal cancer.

Three months after the hepatectomy, the patient exhibited a recurrence at the cut surface of the residual liver. He and his family did not hope further surgery. Although he received chemotherapy using 5-FU for the local recurrence, he died 2 years and 9 mo after the second hepatectomy.

## DISCUSSION

Liver metastases occur in 25%-35 % of patients with colorectal cancer[1]. Surgical resection has become a recognized curative treatment[1,5,6]. However, the rate of intrahepatic recurrence has been reported to range from 16% to 28%[5,7], and 9%-25% of these arise at the surgical margin[8,9]. Okano *et al*[3] reported that 42% of patients who have undergone hepatectomy for colorectal liver metastases exhibit bile duct invasion, 12% of which exhibit macroscopic invasion. Among these metastases, an intrabiliary extension pattern is rare. Kubo *et al*[4] reported that 3.7% of resected colorectal liver metastases are extended predominantly along the bile duct without forming an extrabiliary mass. The prognosis of patients with macroscopic bile duct invasion is better than that of patients with microscopic intraluminal invasion, which tends to aggressively involve the Glisson's triad[3,4].

In the present case, we initially considered the cystic lesion to be a post-operative collection of inflammatory fluid. However, the lesion turned out to be a cystic tumor including mucin produced by the remnant cancer cells. Cancer cells and mucin obstructing the IHBD, caused dilatation of the peripheral bile ducts. Microscopically, about 10% of metastatic tumors from colorectal carcinoma exhibit mucinous features[2]. Furthermore, the frequencies of incidence of localized IHBD dilatation caused by metastatic liver cancer and cystic degeneration of metastatic tumor have been reported to be 6%[10] and 2%-4%, respectively[11,12]. Therefore, the present case was considered to be a very rare case and preoperative diagnosis could not be confirmed by imaging examination alone. On the other hand, it is known that cholangiocellular carcinoma (CCC) is often accompanied with dilatation of the IHBD, because intrahepatic CCC exhibits bile duct extension, either through infiltration into the periductal tissues or appearing as a cast-like growth into the ductal lumen[13]. Furthermore, CCC sometimes exhibits mucin production. Thus, the growth of colorectal metastases with bile duct invasion can be indistinguishable from that of less aggressive CCC, and it is sometimes difficult to make a differential diagnosis histologically. To discriminate liver metastases from primary CCC, immunostaining for CK7 and CK20 can be very useful. A CK20-positive and CK7-negative pattern is highly characteristic of liver metastases from colorectal cancer, compared with most

adenocarcinomas including CCC that are usually CK20-negative and CK7-positive[14-16]. In the present case, this immunohistochemical finding allowed us to make a definite diagnosis.

Our patient has survived for more than two-years, in spite of local recurrence. This may be a consequence of the less aggressive features of this tumor, which exhibits macroscopic bile duct extension[3,4]. However, because this invasion pattern tends to make the cut ends positive for cancer cells, it appears that it is necessary to examine the cut end of Glisson's triad by stamp cytology or frozen section pathology. Actually, during the 2nd hepatectomy, mucinous bile was found in the bile duct. However, since the tumor was a metastatic colorectal cancer rather than a CCC, partial hepatectomy was a procedure of choice for us. For colorectal metastases, we usually perform partial hepatectomy with a sufficient margin rather than anatomical resection if possible. Recently, Pawlik *et al*[9] reported that the width of a negative surgical margin does not affect survival, recurrence risk, or site of recurrence. However, after having experienced our present case, anatomical hepatic resection may be a treatment of choice for metastatic liver tumor with bile duct extension to avoid the potential for a positive surgical margin. Further studies are necessary to develop a suitable surgical procedure for this unusual pattern of liver metastasis.

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**S- Editor** Wang J **L- Editor** Wang XL **E- Editor** Bi L

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*World J Gastroenterol* 2006 August 14; 12(30): 4922-4926
***World Journal of Gastroenterology*** ISSN 1007-9327


*CASE REPORT*

# Obstructive jaundice caused by secondary pancreatic tumor from malignant solitary fibrous tumor of pleura: A case report

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Received: 2006-03-29 Accepted: 2006-04-24

## Abstract

A 77-year-old man on systemic chemotherapy against postoperative bilateral multiple lung metastases of malignant solitary fibrous tumor of the pleura suffered from pruritus and jaundice. Blood examination showed elevated levels of hepatobiliary enzymes. Abdominal computed tomography showed a tumor with peripheral enhancement in the pancreatic head, accompanied with the dilatation of intra- and extra-hepatic bile ducts. He was diagnosed as having obstructive jaundice caused by a pancreatic head tumor. The pancreatic head tumor was presumably diagnosed as the metastasis of malignant solitary fibrous tumor of the pleura, because the findings on the pancreatic head tumor on abdominal CT were similar to those on the primary lung lesion of malignant solitary fibrous tumor of the pleura. The pancreatic tumor grew rapidly after the implantation of metallic stent in the inferior part of the common bile duct. The patient died of lymphangitis carcinomatosa of the lungs. Autopsy revealed a tumor that spread from the pancreatic head to the hepatic hilum. Microscopically, spindle-shaped cells exhibiting nuclear atypicality or division together with collagen deposition were observed. Immunohistochemically the pancreatic head tumor cells were negative for staining of α-smooth muscle actin (α-SMA) or CD117, but positive for vimentin, CD34 and CD99. These findings are consistent with those on malignant solitary fibrous tumor of the pleura. We report the first case of obstructive jaundice caused by a secondary pancreatic tumor from malignant solitary fibrous tumor of the pleura.



**Key words**: Malignant solitary fibrous tumor of the pleura; Secondary pancreatic tumor; Obstructive jaundice

Yamada N, Okuse C, Nomoto M, Orita M, Katakura Y, Ishii T, Shinmyo T, Osada H, Maeda I, Yotsuyanagi H, Suzuki M, Itoh F. Obstructive jaundice caused by secondary pancreatic tumor from malignant solitary fibrous tumor of pleura: A case report. *World J Gastroenterol* 2006; 12(30): 4922-4926

http://www.wjgnet.com/1007-9327/12/4922.asp

## INTRODUCTION

Solitary fibrous tumor of the pleura (SFTP) is a neoplasm derived from mesenchymal cells located in the submesothelial lining of the pleural space, predominantly composed of spindle-shaped cells in combination with collagen deposition[1]. SFTP comprises approximately 5% of primary pleural tumors following malignant mesothelioma[2]. Seven percent to 13% of SFTPs are considered to be malignant neoplasms, of which 41% to 63% recur in the pleura or lung or metastasize to the liver, brain, spleen, adrenal gland and other organs[1,2]. However, no metastasis to the pancreatic head with obstructive jaundice has been reported. Here, we report the first case of obstructive jaundice caused by a secondary pancreatic tumor from malignant SFTP.

## CASE REPORT

The patient was a 77-year-old Japanese man. In July 2001, he was found to have an extrapleural tumor projecting from the right diaphragm by mass screening chest roentgenography. Partial resections of the right diaphragm and lower right lobe of the lungs were carried out in July 2002,

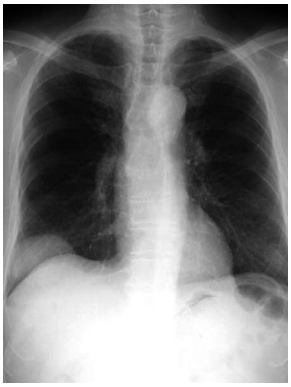

**Figure 1** An extrapleural tumor projecting from the right diaphragm observed on chest roentgenography.

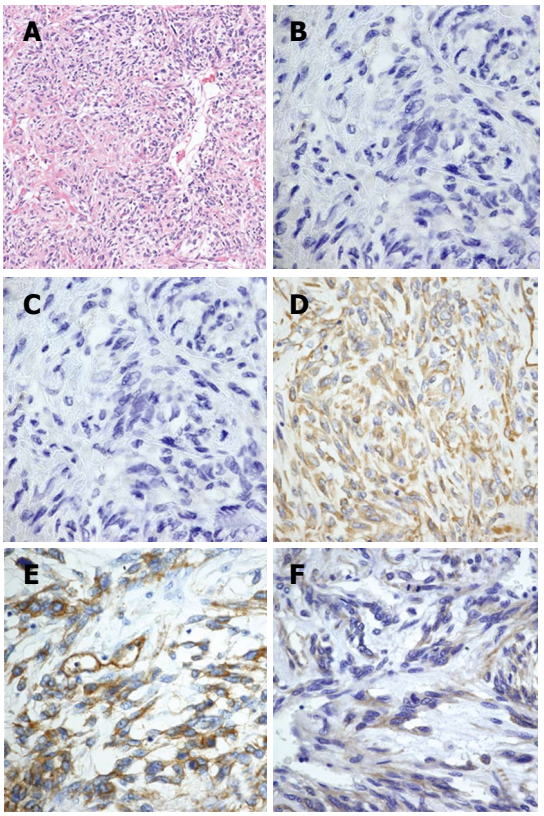


**Figure 2** Spindle-shaped cells exhibiting nuclear atypicality or division together with collagen deposition (hematoxylin and eosin staining) (**A**), tumor cells negative for SMA (**B**) or CD117 (c-kit) (**C**) and positive for vimentin (**D**), CD34 (**E**) and CD99 (**F**) on histological examination.

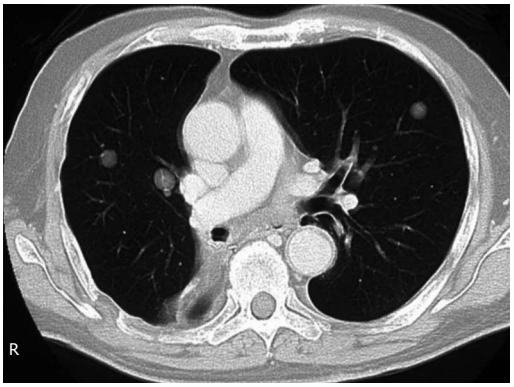


**Figure 3** Multiple nodular lesions in the bilateral lung fields observed on chest computed tomography (CT).

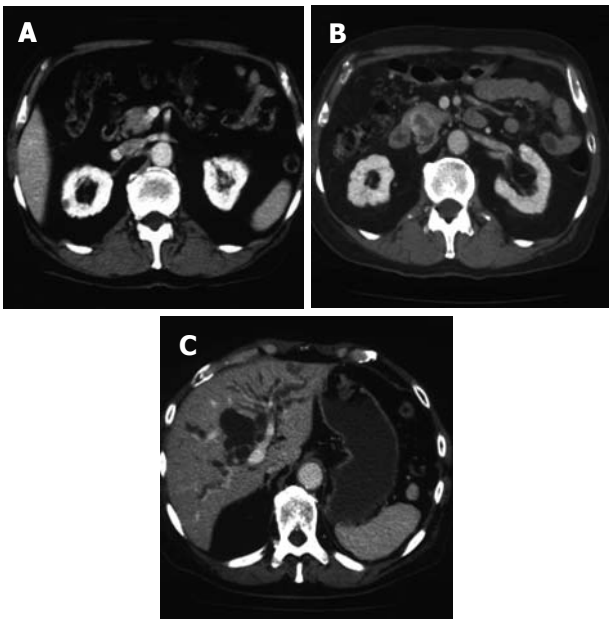


**Figure 4** The pancreatic head tumor not detected by preoperative abdominal CT (**A**), a 25 mm × 17 mm tumor in the pancreatic head detected by abdominal CT with its periphery enhanced by contrast material in the delayed phase (**B**) and dilatation of intra- and extra-hepatic bile ducts observed (**C**).

because of the rapid tumor growth (Figure 1). The tumor in the resected specimens was composed of nonorganized, spindle-shaped cells exhibiting nuclear atypicality or division together with collagen deposition. Immunohistochemical staining showed that the tumor cells were negative for α-SMA or CD117 (c-Kit) but positive for vimentin, CD34 and CD99 (Figures 2A-F). He was diagnosed as having a solitary fibrous tumor of the pleura (SFTP).

In September 2003, multiple metastases were found in the bilateral lung fields and he received systemic chemotherapy with gemcitabine (GEM) and cisplatin (CDDP). Two courses of chemotherapy with GEM and CDDP temporarily reduced the tumor size in the lung.

In mid-April 2004, he experienced pruritus and showed skin jaundice. He was found to have liver dysfunction. He was admitted to our hospital for further evaluation and treatment.

His medical history before 2001 was uneventful. He had a history of daily alcohol intake for 50 years and daily smoking for 40 years.

On the day of admission, he was alert. His body temperature was 35.4℃. His bulbar conjunctiva and skin were icteric. There was a surgical scar on his right lateral thorax. His liver was soft and palpable 3 cm below the umbilical margin. No tumor was palpated in the abdomen. A moveable and elastic tumor 2 cm in diameter was palpable in his right buttock.

The levels of hepatobiliary enzymes and bilirubin were elevated. The levels of tumor markers including carcinoembryonic antigen (CEA) and pancreatic cancer-associated antigen-2 (DUPAN-2) were normal (Table 1). Multiple

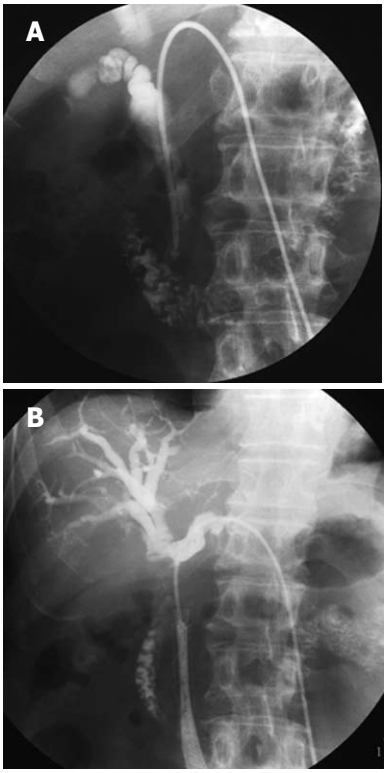


**Figure 5** A marked stenosis of the inferior part of the CBD observed on cholangiography *via* a PTBD tube (**A**), an obstruction from inferior part of CBD to the confluence of the right and left extrahepatic bile ducts involving the whole stent on reperformed cholangiography (**B**) on microscopic examination.

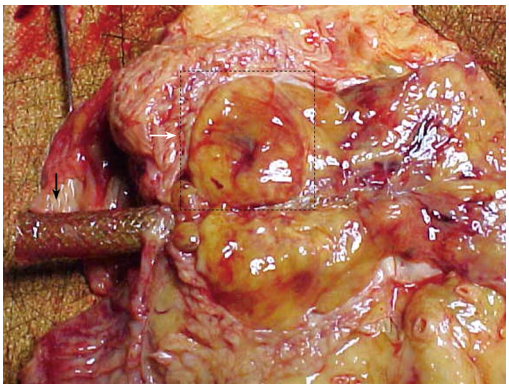

**Figure 6** Macroscopic findings on autopsy. A dissected (120 mm × 30 mm × 20 mm) tumor spreading from the pancreatic head to the hepatic hilum and involving bilateral hepatic ducts. White arrow shows the pancreatic head tumor and black arrow shows the metallic stent.

nodular lesions were observed in the bilateral lung fields by chest computed tomography (CT) (Figure 3). By abdominal CT, a tumor (25 mm × 17 mm) with its periphery enhanced by the contrast material in the delayed phase, was observed in the pancreatic head (Figure 4B). This tumor was not detected by preoperative abdominal CT in 2002 (Figure 4A). The dilatation of intra- and extra-hepatic bile ducts was also observed. Intra-abdominal lymph nodes were not swollen (Figure 4C). Because the radiological findings observed with or without contrast material were similar to those observed in the primary pleural tumor, he was diagnosed as having obstructive jaundice presumably caused by a secondary pancreatic head tumor from malignant SFTP. Percutaneous transhepatic bile duct drainage (PTBD) was performed to improve his jaundice. Cholangiography *via* a PTBD tube showed marked stenosis of the inferior part of the common bile duct (CBD) (Figure 5A). No atypical cell was observed in the bile collected by the tube.

**Table 1 Laboratory findings on admission**

| | | | |
|---|---|---|---|
| **Peripheral blood** | | TP | 6.3 mg/dL |
| WBC | 4900 /μL | Alb | 3.8 mg/dL |
| Hb | 13.8 g/dL | Cr | 0.96 mg/dL |
| Hct | 41.2% | BUN | 13.4 mg/dL |
| Plt | 16.5 × $10^4$ μL | Amy | 107 IU/L |
| **Coagulation** | | FBS | 111 mg/dL |
| PT | 100% | CRP | 0.08 mg/dL |
| APTT | 28.5 (cont: 29.3) | **Tumor Markers** | |
| Fib | 499 mg/dL | CA 19-9 | 16 U/mL |
| **Blood Chemistry** | | CEA | 4.2 ng/mL |
| T-Bil | 4.2 mg/dL | DUPAN-2 | 135 U/mL |
| D-Bil | 3.4 mg/dL | SCC | 1.2 ng/mL |
| AST | 318 IU/L | NSE | 6.6 ng/mL |
| ALT | 444 IU/L | | |
| LDH | 405 IU/L | | |
| ALP | 1860 IU/L | | |
| γ-GTP | 612 IU/l | | |

Because of the bilateral multiple lung and right-buttock tumors, which were considered to have metastasized from the pleura, pancreatoduodenectomy was no longer an indication for treatment.

Thus, a metallic stent was implanted in the inferior part of CBD as a palliative treatment. The levels of hepatobiliary enzymes became normal after the implantation. However, in July 2004, he suffered from fever and jaundice with elevated levels of hepatobiliary enzymes. Abdominal CT demonstrated obstruction of the stent and dilataion of the intrahepatic bile ducts. Furthermore, an obstruction from the inferior part of CBD to the confluence of the right and left extra-hepatic bile ducts involving the whole stent was observed when cholangiography was performed again (Figure 5B). Although a drainage tube was reinserted in the anterior and lateral branches of the intra-hepatic bile ducts, he developed lymphangitis carcinomatosa and died of respiratory failure.

Autopsy was performed with informed consent. Multiple tumors were observed in the bilateral lung. A solitary tumor (25 mm × 15 mm × 15 mm) was observed in his right buttock. A large tumor (120 mm × 30 mm × 20 mm) spread from the pancreatic head to the hepatic hilum and involved bilateral hepatic ducts (Figure 6).

Microscopically, spindle-shaped cells exhibiting nuclear atypicality or division together with collagen deposition were observed (Figure 7A). Immunohistochemically, tumor cells of the pancreatic head were negative for α-SMA or CD117, but positive for vimentin, CD34 and CD99 (Figures 7B-F). These findings were consistent with those on malignant SFTP resected in July 2002.

## DISCUSSION

Malignant SFTP recurs in the pleura or lung or metasta-

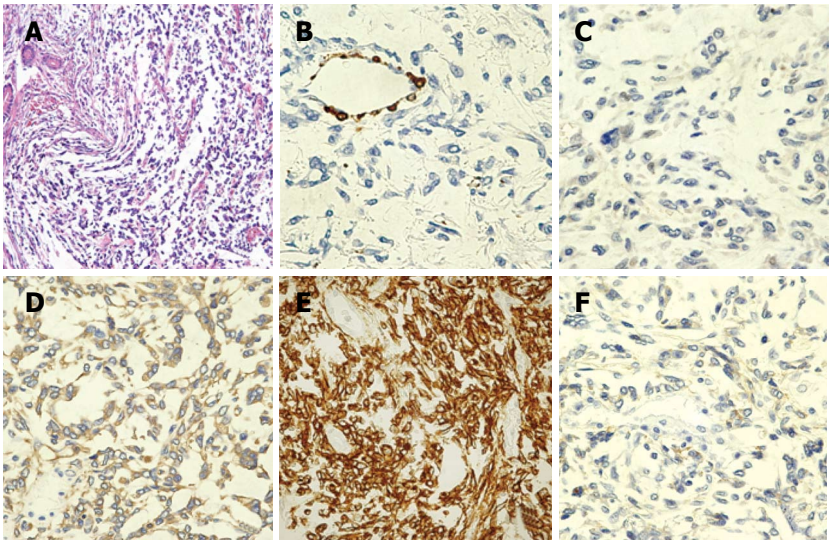


**Figure 7** Spindle-shaped cells exhibiting nuclear atypicality or division together with collagen deposition (hematoxylin and eosin staining) (**A**), tumor cells negative for SMA (**B**) or CD117 (c-kit) (**C**) and positive for vimentin (**D**), CD34 (**E**) and CD99 (**F**) on histological examination. These findings are consistent with those on malignant solitary fibrous tumor of the pleura, resected in July 2002.

sizes to the liver, brain, spleen, adrenal gland and other organs *via* a blood-borne pathway[1-3]. This present patient was considered to have a pancreatic head tumor having metastasized from a malignant SFTP for the following reasons. Multiple metastatic lesions in the bilateral lung and a growing subcutaneous tumor were observed and the findings on the pancreatic head tumor by abdominal CT were similar to those on the primary lung SFTP. The tentative diagnosis was confirmed by the histopathological and immunohistochemical findings on autopsy.

Secondary pancreatic tumor is rare. Nakamura *et al*[4] reported that the incidence of secondary pancreatic tumor is approximately 6% in autopsy studies and increases to approximately 15% in cases of malignant tumors. The lung is the second most common primary site (18%) of secondary pancreatic tumor[4,5]. The histopathological types of this tumor are large cell carcinoma, small cell carcinoma, adenocarcinoma and squamous cell carcinoma[4-7]. Malignant SFTP has never been reported as a cause of secondary pancreatic tumor to date.

In general, secondary pancreatic tumor is caused by blood-borne metastasis, lymphatic metastasis and tumor infiltration from adjacent organs or peritoneal seeding. The secondary pancreatic tumor of this present patient was considered to be caused by blood-borne metastasis because no metastasis in regional lymph nodes was observed during operation or on autopsy, but multiple metastatic lesions in the bilateral lungs and a subcutaneous metastatic tumor in the right buttock were observed.

Although radiation therapy and chemotherapy have been performed against malignant SFTP in addition to resection, they could not achieve sufficient therapeutic efficacy[2]. On the other hand, GEM monotherapy or combination therapy with CDDP is effective against malignant mesothelioma[8].

Chemotherapy with GEM and CDDP, which is effective for patients with malignant mesothelioma, was selected for this patient because the treatment of malignant SFTP with metastasis was not authorized, and both malignant mesothelioma and SFTP originated from the pleura. However, the tumor grew more rapidly even after chemotherapy. Forty-one percent to 63% of malignant SFTPs recur locally or metastasize to other organs[1,2] and 23% of these patients die within 24 mo of diagnosis[9]. Therefore, the establishment of an effective anti-tumor therapy against malignant SFTP is desired.

The leading drug for the novel therapy is imatinib mesylate, which is effective against gastrointestinal stromal tumors (GISTs)[10]. In half of the patients with malignant SFTP, tumor cells have been found positive for c-kit[11]. Therefore, imatinib mesylate, which inhibits kit-thyrosine kinase, may be a promising drug for molecular-target-based therapy. This hypothesis should be verified in future studies.

In summary, we report a rare case of obstructive jaundice caused by a secondary pancreatic tumor from malignant SFTP. Pancreatic metastasis may adversely affect the prognosis of malignant SFTP. Therefore, abdominal images and the levels of hepatobiliary enzymes should be examined regularly.

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**S- Editor** Wang J **L- Editor** Wang XL **E- Editor** Bai SH

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*World J Gastroenterol* 2006 August 14; 12(30): 4927-4929
***World Journal of Gastroenterology*** ISSN 1007-9327


*CASE REPORT*

# Pancreatitis complicating mucin-hypersecreting common bile duct adenoma

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Received: 2006-02-23 Accepted: 2006-03-13

## Abstract

Villous adenomas of the bile ducts are extremely uncommon. We describe a 58-year-old man presenting with clinical signs and laboratory findings of acute pancreatitis and obstructive jaundice. Preoperative investigation demonstrated a dilated papillary orifice with mucus exiting (fish-mouth sign) and a filling defect in the distal common bile duct. He underwent a modified Whipple operation and histological examination of the surgical specimen showed villous adenoma with rich secretion of mucus.



**Key words:** Villous adenoma; Common bile duct; Endoscopic retrograde cholangiopancreatography

Katsinelos P, Basdanis G, Chatzimavroudis G, Karagiannoulou G, Katsinelos T, Paroutoglou G, Papaziogas B, Paraskevas G. Pancreatitis complicating mucin-hypersecreting common bile duct adenoma. *World J Gastroenterol* 2006; 12(30): 4927-4929

http://www.wjgnet.com/1007-9327/12/4927.asp

## INTRODUCTION

Villous adenomas are benign epithelial lesions with malignant potential, which are usually encountered in the colon, less commonly in the small bowel or the ampulla of Vater, and extremely uncommonly in the bile ducts[1]. Since Saxe *et al* first reported a case of villous adenoma of the common bile duct (CBD) in 1988, a total of 17 cases have been reported so far in the literature[1-17].

We present what is to our knowledge, the first case of a mucin-secreting CBD adenoma presenting as acute pancreatitis due to mucus hypersecretion and showing the endoscopic finding of "fish-mouth" in the papilla of Vater.

## CASE REPORT

A 58-year-old man was admitted to the department of Internal Medicine complaining for upper abdominal pain radiating to the back, nausea and vomiting during the last six hours.

Physical examination demonstrated a well nourished man who appeared jaundiced. The abdomen was soft but revealed tenderness in the epigastrium and a movable, firm, non-tender mass in the right upper quadrant, which was felt to be a distended gallbladder. Previous medical history included a 3-mo period of dyspepsia. The patient did not mention previous episodes of fever or weight loss. He had never been abroad, was not on any drug or alcohol abuse and had no history of serious illness.

Laboratory investigations showed a total serum bilirubin level of 51 μmol/L (normal range 0-3 μmol/L), direct bilirubin level of 39 μmol/L, alkaline phosphatase level of 548 U/L (normal range < 120 U/L), alanine transaminase level of 143 U/L (normal range < 45 U/L) and serum amylase level of 1720 U/L (normal range < 30 U/L). The hemogram revealed hemoglobin and hematocrit of 142 g/L and 44.6%, respectively, while white blood cell count was 16 100 /mm$^3$. On abdominal ultrasound and CT the pancreas was edematous, the gallbladder was distended without calculi and the CBD was found to be dilated (12.5 mm), with a nonshadowing tissue mass in its distal end. The patient was treated conservatively with resolution of symptoms.

On seventh day, an endoscopic retrograde cholangiopancreatography (ERCP) was performed and demonstrated a patulous papillary orifice of the major papilla (fish-mouth sign) with mucus exuding from it (Figure 1), a normal pancreatogram and a 2 cm × 1 cm intraluminal filling defect in the distal CBD (Figure 2).

A sphincterotomy was performed and in order to rule out the presence of stone, a balloon was inserted, but nothing was retrieved. A subsequent lithotriptor-basket extraction took a fragment tissue out and a 8.5-French Cotton-Leung type plastic stent of 7 cm was placed.

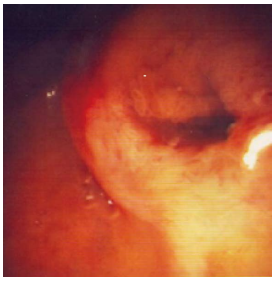

**Figure 1** Endoscopic view showing a dilated papillary orifice exiting mucus.

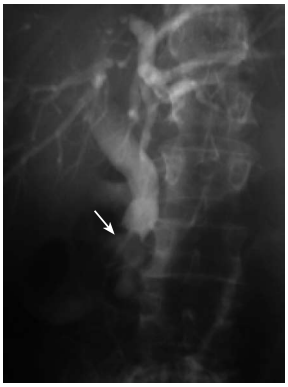

**Figure 2** ERCP demonstrating an intraluminal filling defect in the distal common bile duct. A 8.5Fr stent has been placed.

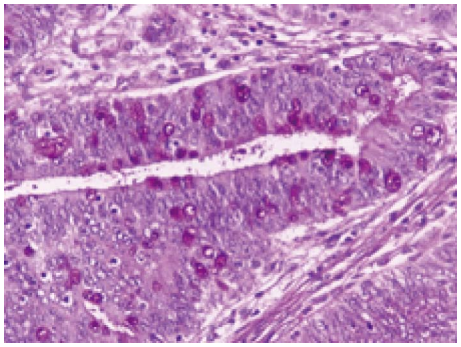

**Figure 3** Surgical specimen showing that the tumor consists of tall, columnar, pseudostratified epithelial cells with elongated or round atypical nuclei. Some of the cells contain large amounts of mucin within the apical cytoplasm (PAS X 400).

Because the histological examination of the retrieved tissue showed that the main mass was a villous adenoma, the mass was thought to be resectable with safety margins. The patient subsequently underwent a modified Whipple procedure. The histopathological examination of the specimen demonstrated that the tumor consisted of finger-like villous or papillary processes containing central thin cords of lamina propria lined by a neoplastic epithelium. The epithelial cells were tall columnar, pseudostratified with elongated or round atypical nuclei. Some of the cells contained large amounts of mucin within the apical cytoplasm. Focally goblet cells lied within the epithelium (Figure 3). No signs of hyperplasia or adenoma of the pancreas were observed in the specimen.

The patient recovered uneventfully and remains in good general condition, six months after discharge.

## DISCUSSION

Adenomas of the bile ducts are divided into papillary adenomas, penduculated adenomas and sessile adenomas, according to the gross configuration of the tumors[18]. This classification replaced an earlier classification of papillomas and adenomas. Villous adenomas are thus classified as frond-like sessile adenomas[3]. Histologically, however, adenomas are classified into tubular, tubulovillous and villous adenomas.

The most common site for villous adenomas in the biliary tree is the CBD. These adenomas usually develop in the distal aspect of the CBD[18]; they are histologically similar to villous adenomas in the ampullary region, gallbladder and intestine and should probably be considered having similar biological behavior. The adenoma to carcinoma sequence is well accepted in the colon and most likely also applies in the ampullary region, gallbladder and bile ducts[19].

Of note, in reviewing the reported cases[1-17] of bile duct villous adenomas, the clinical picture includes painless jaundice, pruritus, upper abdominal pain, cholangitis and dyspepsia. The tumors rarely grow large enough to become palpable because of their site. The degree of jaundice has been described to fluctuate in some reports, which may be attributable to a ball valve effect of the tumor. Preoperative diagnosis or diagnosis without operation was possible in 6 out of 17 cases, mainly in recently reported cases, reflecting the current sophisticated diagnostic and therapeutic techniques of the biliary tract and pancreas. Abdominal US and CT demonstrated dilation of the CBD and an intraductal tumor but were not capable of specifying any further the nature of the lesion. The absence of gallbladder calculi, as well as the absence of acoustic shadowing of the CBD mass in the patients, made the possibility of the lesion being calculus unlikely. In addition, in the absence of gallbladder sludge, sludge in the CBD is unusual. Moreover, differentiation of a villous adenoma from other nonshadowing solid lesions, such as blood clots, nonshadowing calculi, lipomas, fibromas, or carcinoid tumors on the basis of US and CT findings is difficult[5]. Echoendoscopy is a well proven technique with higher image resolution and greater diagnostic sensitivity than conventional imaging methods and would be useful to identify the nature of the lesion and exclude an invasive component.

It must be emphasized that two cases of coexisting biliary tract and intestinal polyps have been reported[20,21]. It has been proposed that bile duct adenomas are part of the spectrum of generalized gastrointestinal polyposis. Some authors[20-23] have suggested that upper gastrointestinal surveillance of patients with familiar adenomatous polyposis should routinely include imaging of the biliary tract.

Our case is both unique and challenging in that the CBD adenoma had close resemblance to intraductal papillary mucinous tumors (IPMT) of the pancreas. More specifically, it was composed of secreting cells, with hypersecretion of mucin. The hypersecreted viscous mucin led to obstruction of the papillary orifice, thus increasing the pancreatic intraductal pressure and activating the cascade of mechanism of pancreatitis. Moreover, the "fish-mouth" sign in the major papilla is an endoscopic finding

which has been discussed in mucin-secreting pancreatic tumors. As both lesions are encountered premalignant, complete resection of both of them is considered mandatory. Therefore, we suggest that the term IPMT of the bile ducts could be used to describe this rare condition.

In conclusion, our case reflects the need to consider a wide differential in patients presenting with acute pancreatitis and jaundice, especially in the absence of cholelithiasis and alcohol abuse.

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**S- Editor** Wang J **L- Editor** Lakatos PL **E- Editor** Liu WF

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*World J Gastroenterol* 2006 August 14; 12(30): 4930-4931
***World Journal of Gastroenterology*** ISSN 1007-9327


*LETTERS TO THE EDITOR*

# Therapeutic endoscopic retrograde cholangiopancreatography and related modalities have many roles in hepatobiliary hydatid disease

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Received: 2006-04-21 Accepted: 2006-05-22

## Abstract

The authors report their experience about 8 cases of intrabiliary rupture of hepatobiliary hydatid disease, and add an algorithm for treatment. To our opinion, the use of diagnostic and therapeutic endoscopic retrograde cholangiopancreatography (ERCP) in the management of hepatobiliary hydatid disease was not stated properly in their proposed algorithm. According to the algorithm, the use of ERCP and related modalities was only stated in the case of postoperative biliary fistulae. We think that postoperative persistant fistula is not a sole indication, there are many indications for ERCP and related techniques namely sphincterotomy, extraction, nasobiliary drainage and stenting, in the treatment algorithm before or after surgery.



**Key words:** Therapeutic endoscopic retrograde cholangio pancreatography; Hepatobiliary; Hydatid

Özaslan E. Therapeutic endoscopic retrograde cholangiopancreatography and related modalities have many roles in hepatobiliary hydatid disease. *World J Gastroenterol* 2006; 12(30): 4930-4931

http://www.wjgnet.com/1007-9327/12/4930.asp

## TO THE EDITOR

We have read with great interest the article titled "Intrabiliary rupture: an algorithm in the treatment of controversial complication of hepatic hydatidosis"[1]. The authors reported their experience about 8 cases of intrabiliary rupture of hepatobiliary hydatid disease, and added an algorithm for treatment. To our opinion, the use of diagnostic and therapeutic endoscopic retrograde cholangiopancreatography (ERCP) in the management of hepatobiliary hydatid disease was not stated properly in their proposed algorithm. According to the algorithm, in a case of preoperative suspicion of intrabiliary rupture due to various reasons, such as cystic content in common bile duct (CBD), dilated CBD and obstructive jaundice, surgery was proposed without prior to ERCP. The use of ERCP and related modalities was only stated in the case of postoperative biliary fistula. Many reports[2-4] including ours[5,6] are published in English literature about the use of ERCP in the management of hydatid disease. Our former report[5] has reviewed a total of 294 cases, after collecting 273 cases in 26 articles and 6 abstracts from literature and adding 21 cases of our own experience. Considering the current literature[7,8] and our experience, we think that postoperative persistant fistula is not a sole indication, there are many indications for ERCP and related techniques namely sphincterotomy, extraction, nasobiliary drainage and stenting, in the treatment algorithm before or after surgery.

ERCP in the preoperative period[2] Ⅰ- defines the cystobiliary relationship to help in surgery planning, Ⅱ- permits evaluation of acute conditions like cholangitis and obstruction so that subsequent surgery can be performed on an elective basis, Ⅲ- may give permanent cure specifically in cases of frank intrabiliary rupture if evacuation of biliary tract and cystic cavity is manageable, and Ⅳ- when combined with preoperative endoscopic sphincterotomy may decrease the incidence of the development of postoperative external fistulae. While the first three statements have been studied extensively, the fourth statement may warrant further studies to clarify the criteria of selection of appropriate cases. The only study regarding this issue performed by Galati *et al*[7], reported a significant decrease in postoperative fistulae in cases that underwent selective preoperative ERCP (3.8% *versus* 7.4%).

ERCP in the postoperative period[2] Ⅰ- can help to clarify the causes of ongoing or recurrent symptoms or laboratory abnormalities, Ⅱ- may help to resolve the obstruction or cholangitis due to residual material in biliary ducts, Ⅲ- may provide the chance to manage postoperative external biliary fistulae, and Ⅳ- may be a realistic solution for secondary biliary strictures[8].

Since hydatid disease is a serious public health problem despite the use of various kinds of preventive measures,

we greatly appreciate every kind of studies regarding the issue to solve the controversions. Endoscopic therapy should be incorporated into the other treatment options including surgery, percutaneous measures and chemotherapy with benzimidazole compounds. The exact place of each therapeutic modality in a particular case should be decided on the basis of expanding current literature.

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*World J Gastroenterol* 2006 August 14; 12(30): 4932
***World Journal of Gastroenterology*** ISSN 1007-9327


*ACKNOWLEDGMENTS*

# Acknowledgments to Reviewers of *World Journal of Gastroenterology*

Many reviewers have contributed their expertise and time to the peer review, a critical process to ensure the quality of *World Journal of Gastroenterology*. The editors and authors of the articles submitted to the journal are grateful to the following reviewers for evaluating the articles (including those were published and those were rejected in this issue) during the last editing period of time.

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*World J Gastroenterol* 2006 August 14; 12(30): 4933
***World Journal of Gastroenterology*** ISSN 1007-9327


# Meetings

## MAJOR MEETINGS COMING UP

First Biennial Congress of the Asian-Pacific Hepato-Pancreato-Biliary Association
March, 2007
Fukuoka, Japan
http://www.congre.co.jp/1st-aphpba

American College of Gastroenterology Annual Scientific
20-25 October 2006
Las Vegas, NV

14th United European Gastroenterology Week, UEGW
21-25 October 2006
Berlin, Germany

APDW 2006: Asian Pacific Digestive Week 2006
26-29 November 2006
Lahug Cebu City, Philippines

## EVENTS AND MEETINGS IN THE UPCOMING 6 MONTHS

Falk Symposium 151: Emerging Issues in Inflammatory Bowel Diseases
24-25 March 2006
Sydney - NSW
Falk Foundation e.V.
symposia@falkfoundation.de

10th International Congress of Obesity
3-8 September 2006
Sydney
Event Planners Australia
enquiries@ico2006.com
www.ico2006.com

Easl 2006 - the 41st annual
26-30 April 2006
Vienna, Austria
Kenes International

Prague hepatology 2006
14-16 September 2006
Prague
Foundation of the Czech Society of Hepatology
veronika.revicka@congressprague.cz
www.czech-hepatology.cz/phm2006

12th International Symposium on Viral Hepatitis and Liver Disease
1-5 July 2006
Paris
MCI France
isvhld2006@mci-group.com
www.isvhld2006.com

Falk Symposium 152: Intestinal Disease Part I, Endoscopy 2006 - Update and Live Demonstration
4-5 May 2006
Berlin
Falk Foundation e.V.
symposia@falkfoundation.de

Falk Symposium 153: Intestinal Disease Part II, Immunoregulation in Inflammatory Bowel Disease - Current Understanding and Innovation
6-7 May 2006
Berlin
Falk Foundation e.V.
symposia@falkfoundation.de

ILTS 12th Annual International Congress
3-6 May 2006
Milan
ILTS
www.ilts.org

Internal Medicine: Gastroenterology
22 July 2006-1 August 2006
Amsterdam
Continuing Education Inc
jbarnhart@continuingeducation.net

6th Annual Gastroenterology And Hepatology
15-18 March 2006
Rio Grande
Office of Continuing Medical Education
cmenet@jhmi.edu
www.hopkinscme.net

World Congress on Gastrointestinal Cancer
28 June 2006-1 July 2006
Barcelona, Spain
c.chase@imedex.com

International Conference on Surgical Infections, ICSI2006
6-8 September 2006
Stockholm
European Society of Clinical Microbiology and Infectious Diseases
icsi2006@stocon.se
www.icsi2006.se/9/23312.asp

7th World Congress of the International Hepato-Pancreato-Biliary Association
3-7 September 2006
Edinburgh
Edinburgh Convention Bureau
convention@edinburgh.org
www.edinburgh.org/conference

Society of American Gastrointestinal Endoscopic Surgeons
26-29 April 2006
Dallas - TX
www.sages.org

Digestive Disease Week 2006
20-25 May 2006
Los Angeles
www.ddw.org

Annual Postgraduate Course
25-26 May 2006
Los Angeles, CA
American Society of Gastrointestinal Endoscopy
www.asge.org/education

American Society of Colon and Rectal Surgeons
3-7 June 2006
Seatlle - Washington
www.fascrs.org

## EVENTS AND MEETINGS IN 2006

10th World Congress of the International Society for Diseases of the Esophagus
22-25 February 2006
Adelaide
isde@sapmea.asn.au
www.isde.net

Falk Symposium 151: Emerging Issues in Inflammatory Bowel Diseases
24-25 March 2006
Sydney - NSW
Falk Foundation e.V.
symposia@falkfoundation.de

10th International Congress of Obesity
3-8 September 2006
Sydney
Event Planners Australia
enquiries@ico2006.com
www.ico2006.com

Easl 2006 - the 41st annual
26-30 April 2006
Vienna, Austria
Kenes International

VII Brazilian Digestive Disease Week
19-23 November 2006
www.gastro2006.com.br

International Gastrointestinal Fellows Initiative
22-24 February 2006
Banff, Alberta
Canadian Association of Gastroenterology
cagoffice@cag-acg.org
www.cag-acg.org

Canadian Digestive Disease Week
24-27 February 2006
Banff, Alberta
Digestive Disease Week Administration
cagoffice@cag-acg.org
www.cag-acg.org

Prague Hepatology 2006
14-16 September 2006
Prague
Foundation of the Czech Society of Hepatology
veronika.revicka@congressprague.cz
www.czech-hepatology.cz/phm2006

12th International Symposium on Viral Hepatitis and Liver Disease
1-5 July 2006
Paris
MCI France
isvhld2006@mci-group.com
www.isvhld2006.com/

Falk Seminar: XI Gastroenterology Seminar Week
4-8 February 2006
Titisee
Falk Foundation e.V.
symposia@falkfoundation.de

European Multidisciplinary Colorectal Cancer Congress 2006
12-14 February 2006
Berlin
Congresscare
info@congresscare.com
www.colorectal2006.org

Falk Symposium 152: Intestinal Disease Part I, Endoscopy 2006 - Update and Live Demonstration
4-5 May 2006
Berlin
Falk Foundation e.V.
symposia@falkfoundation.de

Falk Symposium 153: Intestinal Disease Part II, Immunoregulation in Inflammatory Bowel Disease - Current Understanding and Innovation
6-7 May 2006
Berlin
Falk Foundation e.V.
symposia@falkfoundation.de

14th United European Gastroenterology Week
21-25 October 2006
Berlin
United European Gastroenterology Federation
www.uegw2006.de

World Congress on Controversies in Obesity, Diabetes and Hypertension
25-28 October 2006
Berlin
comtec international
codhy@codhy.com
www.codhy.com

Asia Pacific Obesity Conclave
1-5 March 2006
New Delhi
info@apoc06.com
www.apoc06.com/

ILTS 12th Annual International Congress
3-6 May 2006
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ILTS
www.ilts.org

XXX Panamerican Congress of Gastroenterology
11-16 November 2006
Cancun
www.panamericano2006.org.mx

Internal Medicine: Gastroenterology
22 July 2006-1 August 2006
Amsterdam
Continuing Education Inc
jbarnhart@continuingeducation.net

6th Annual Gastroenterology And Hepatology
15-18 March 2006
Rio Grande
Office of Continuing Medical Education
cmenet@jhmi.edu
www.hopkinscme.net

Hepatitis 2006
25 February 2006-5 March 2006
Dakar
hepatitis2006@mangosee.com
mangosee.com/mangosteen/hepatitis2006/hepatitis2006.htm

World Congress on Gastrointestinal Cancer
28 June 2006-1 July 2006
Barcelona, Spain
c.chase@imedex.com

International Conference on Surgical Infections, ICSI2006
6-8 September 2006
Stockholm
European Society of Clinical Microbiology and Infectious Diseases
icsi2006@stocon.se
www.icsi2006.se/9/23312.asp

5th International Congress of The African Middle East Association of Gastroenterology
24-26 February 2006
Sharjah
InfoMed Events
infoevent@infomedweb.com
www.infomedweb.com

7th World Congress of the International Hepato-Pancreato-Biliary Association
3-7 September 2006
Edinburgh
Edinburgh Convention Bureau
convention@edinburgh.org
www.edinburgh.org/conference

13th International Symposium on Pancreatic & Biliary Endoscopy
20-23 January 2006
Los Angeles - CA
laner@cshs.org

2006 Gastrointestinal Cancers Symposium
26-28 January 2006
San Francisco - CA
Gastrointestinal Cancers Symposium
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giregistration@jspargo.com

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Digestive Disease Week 2006
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American Society of Colon and Rectal Surgeons
3-7 June 2006
Seatlle - Washington
www.fascrs.org

71st ACG Annual Scientific and Postgraduate Course
20-25 October 2006
Venetian Hotel, Las Vegas, Neveda
The American College of Gastroenterology

AASLD 57th Annual - The Liver Meeting™
27-31 October 2006
Boston, MA
AASLD

New York Society for Gastrointestinal Endoscopy
13-16 December 2006
New York
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## EVENTS AND MEETINGS IN 2007

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Barcelona
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meetings@imedex.com

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***World Journal of Gastroenterology*** ISSN 1007-9327


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*Chinese journal article (list all authors and include the PMID where applicable)*

2 **Lin GZ**, Wang XZ, Wang P, Lin J, Yang FD. Immunologic effect of Jianpi Yishen decoction in treatment of Pixu-diarrhoea. *Shijie Huaren Xiaohua Zazhi* 1999; **7**: 285-287

*In press*

3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci U S A* 2006; In press

*Organization as author*

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

*Both personal authors and an organization as author*

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

*No author given*

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

*Volume with supplement*

7 **Geraud G**, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment of migraine and in comparison with sumatriptan. *Headache* 2002; **42** Suppl 2: S93-99 [PMID: 12028325]

*Issue with no volume*

8 **Banit DM**, Kaufer H, Hartford JM. Intraoperative frozen section analysis in revision total joint arthroplasty. *Clin Orthop Relat Res* 2002; (**401**): 230-238 [ PMID: 12151900 ]

*No volume or issue*

9 Outreach: bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804 ]

**Books**

*Personal author(s)*

10 **Sherlock S**, Dooley J. Diseases of the liver and billiary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

*Chapter in a book (list all authors)*

11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

*Author(s) and editor(s)*

12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wieczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

*Conference proceedings*

13 **Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ Cell Tumour Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

*Conference paper*

14 **Christensen S**, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

**Electronic journal** (list all authors)

**Morse SS**. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http//www.cdc.gov/ncidod/EID/eid.htm

**Patent** (list all authors)

16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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Present as mean ± SD or mean ± SE.

### *Statistical expression*

Express *t* test as *t* (in italics), *F* test as *F* (in italics), chi square test as $\chi^2$ (in Greek), related coefficient as *r* (in italics), degree of freedom as γ (in Greek), sample number as *n* (in italics), and probability as *P* (in italics).

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Use SI units. For example: body mass, m (B) = 78 kg; blood pressure, *p*(B) = 16.2/12.3 kPa; incubation time, t (incubation) = 96 h, blood glucose concentration, c (glucose) 6.4 ± 2.1 mmol/L; blood CEA mass concentration, ρ (CEA) = 8.6 24.5 μg/L; $CO_2$ volume fraction, 50 mL/L $CO_2$ not 5% $CO_2$; likewise for 40 g/L formaldehyde, not 10% formalin; and mass fraction, 8 ng/g, *etc.* Arabic numerals such as 23, 243, 641 should be read 23 243 641.

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Signed: _____

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